DOCTORAL THESIS

Mechanisms of axonal regeneration after central nervous system injury

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MECHANISMS OF AXONAL REGENERATION

AFTER CENTRAL NERVOUS SYSTEM INJURY

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DECLARATION

I, Rita Juneja, confirm that the work presented in this thesis has been performed and interpreted solely by myself except where explicitly identified to the contrary. I confirm that this work is submitted in partial fulfilment for the degree of PhD in neuroscience and has not been submitted elsewhere in any other form for the fulfilment of any other degree of qualification.

.....

15 August 2012

ABBREVIATIONS

BSF2	**Bottenstein and Sato's Medium**
BDNF	**Brain derived neurotrophic factor**
CAM	**Cell Adhesion Molecule**
cAMP	**Cyclic Adenosine Monophosphate**
chABC	**Chondroitinase ABC**
CNS	**Central Nervous System**
CNTF	**Ciliary neurotrophic factor**
CREB	**cAMP response element-binding protein**
CSPG	**Chondroitin Sulfate Proteoglycans**
DCC	**Deleted in Colorectal Cancer**
DRG	**Dorsal root ganglion**
ECM	**Extracellular matrix**
GDNF	**Glial cell derived nerve growth factor**
GPI	**Glycosylphosphatidylinositol**
L-15	**Leibovitz's -15 Medium**
LIF	**Leukemia inhibitory factor**
MAG	**Myelin Associated glycoprotein**
MAP	**Microtubule-Associated Protein**
MAS	**Minimal Access Surgery**
MIS	**Minimal Access Surgery**
NCAM	**Neural Cell Adhesion Molecule**
NGF	**Nerve growth factor**
NSC	**Neural Stem Cell**
NT-3	**Neurotrophin - 3**
NT-4	**Neurotrophin - 4**

NT-5	**Neurotrophin - 5**
NT-6	**Neurotrophin - 6**
NT-7	**Neurotrophin - 7**
NTF	**Neurotrophic factor**
NTR	**Neurotrophin**
OMgp	**Oligodendrocyte Myelin Glycoprotein**
PBS	**Phosphate Buffered Saline**
PFA	**Paraformaldehyde**
PNS	**Peripheral Nervous System**
RGC	**Retinal Ganglion Cell**
SCI	**Spinal Cord Injury**
TBI	**Traumatic Brain Injury**
VAS	**Visual Assisted Surgery**

ABSTRACT

It has been long stated that the majority of neurons in the adult mammalian CNS have a very limited capacity for spontaneous axonal regeneration after injury. However, the situation is quite different in lower vertebrates such as teleost fish and amphibians. These species have the ability to regenerate severed CNS axons and re-establish connections to form synaptic contacts to the suitable target sites, allowing functional recovery. Recent research findings have indicated that the failure of successful axonal regeneration in the matured mammalian CNS is not simply due to intrinsic properties of neurons, but depends greatly on the post-lesion environments which are known to be non-permissive for the regrowing of axons. There are numbers of factors present in the injured CNS environment that impede the regrowth of injured axons. There are also factors in the CNS environment which act as inhibitory or promoting factors. Imbalances in these factors within the CNS environment all contribute to the collapse of axon growth. These inhibitory molecules include MAG, Nogo, OMgp and CSPG. Neurotrophic factors and cell adhesion molecules are factors required to promote successful axon regeneration and path finding in the CNS.

The adult zebrafish was used as a model for investigating neurite regeneration and to determine how different substrates and promoting factors contribute to the neurite outgrowth in all four studies. The aim of this study was to investigate neurite growth after CNS injury in adult zebrafish. This study was divided into four sections. 1) Isolating and culturing adult zebrafish neurons in tissue culture system. 2) Using neurotrophic factors to determine the effect on neurite growth. 3) Using different substrates coating on coverslips to grow the neurons. 4) The co-culturing of adult zebrafish neurites on rat tissue sections.

The first study examined the growth of adult zebrafish neurons in a tissue culture system using two different media: L-15 and BSF2. This was a comparative study to determine the most suitable medium and environment (CO_2 and enzymatic digestion) for zebrafish neuronal cells. The cells were grown in both media, with different percentages of CO_2 and the presence or absence of enzymatic digestion. Disassociated neurons obtained from adult zebrafish *(Danio Rerio)* brain were successfully grown for at least a week. It was determined that BSF2 was the most suitable medium for adult zebrafish neurons using 1.5% CO_2. Furthermore, the addition of collagenase to dissociate the cell did not affect the neurite growth.

Aside from the cellular environment, the presence of neurotrophic factors may be important and necessary for neuronal cell growth. Hence, in the second study, the experiment was performed to examine whether or not neurotrophic factors played an essential role in promoting growth. Adult zebrafish neurons were grown in the presence or absence of various neurotrophic factors. These neurotrophic factors included BDNF, NGF, LIF, and NT-3. The neuronal cells were treated with these factors for a period of fourteen days. The outcome of the study revealed that LIF had the greatest enhancing effects on neurite outgrowth. This study clearly showed that neurotrophic factors play an important role in neurite growth enhancement from adult zebrafish CNS neurons.

In the third study, a substrate assessment was performed to verify the contribution of cell adhesion and attachment mechanisms to neuronal cell growth. In this particular experiment, the substrates were used either individually or in combination. The substrate or substrate combinations examined included collagen, fibronetic+gelatin,

poly-L-lysine, poly-D- lysine, laminin and poly- L- lysine + Laminin. These substrates were applied to the coverslips prior to cell culturing. Combinations of fibronectin-gelatin coated coverslips were found to result in the greatest amount of neurite regeneration from adult zebrafish CNS neurons.

The final study examined the ability of the adult zebrafish CNS neuronal cells to grow on various rat tissue substrates. Tissue sections from an adult rat brain (cerebellum and cortex) were prepared using a cryostat and used as a substrate on which neurons from adult zebrafish were grown. The tissue sections were visualised with anti- GFAP antibody and the neurons were visualised using anti GAP-43 antibody. It was found that adult zebrafish CNS neurons were capable of extending their neurites on these tissue sections. Previous studies in the literature have shown that mammalian CNS neurons are incapable of extending neurites on such tissue sections. These results, therefore, demonstrated that adult zebrafish CNS neurons did not find such tissue sections as being inhibitory to their neurite regeneration capabilities. It was concluded that such neurons may not possess the receptors to the inhibitory molecules normally found in the mammalian CNS tissues examined in this study. This may be an explanation for the successful regeneration of axons obtained from lower vertebrate species. This study clearly demonstrates that adult zebrafish neurons can be isolated, cultured and assessed for neuronal regeneration. The techniques employed in this study may assist in advancing research on neuronal repair in vivo as well as serving as a platform for future studies. Furthermore, the implications of this finding may be helpful in promoting the successful regeneration of axons from mammalian CNS neurons by blocking or

removing receptors that respond to inhibitory signals in the environment provided by the mature mammalian CNS.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 HISTORICAL ASPECT OF CENTRAL NERVOUS INJURY

Our understanding of the historical aspect of spinal cord Injury could be traced back to ancient Greece and Egypt in 2,600 BC (Sanan and Rengachary, 1996). In those times, meat and honey were used to treat cervical sprain. Meat was tied to the neck and honey was used as medicine to rub over the injury. Traumatic spinal injury and paralysis were also mentioned in the recordings but no method of treatment was provided (Lifshutz and Colohan, 2004, Pearce, 2008). The importance of the spinal cord, however, was acknowledged by Hippocrates in 400 BC (Goodrich, 2004). The spinal cord was known as 'spinal marrow' and the spinal marrow was to be protected from injuries. Patients with spinal cord injury were tied to rungs and placed upside down and they would be shaken sturdily to reduce the spinal deformities. However, this method was highly ineffective in repairing spinal cord injury (Marketos and Skiadas, 1999a, Lifshutz and Colohan, 2004, Naderi et al., 2004, Filler, 2007, Pearce, 2008).

The idea of a central nervous system was launched by the Roman physician, Galen, in 200 AD (Naderi et al., 2004). He pointed out that the spinal cord connects the brain to the limbs and back (Marketos and Skiadas, 1999b, Bennett and Hacker, 2002). Later in the 17th century, the concept of surgery was introduced by Paulus of Aegina who believed that spinal column surgery could be used to remove bone fragments which may solve/prevent the problem of paralysis. The problems with performing simple surgery on the spinal cord were pointed out by scholars in the 10th Century. There were various suggestions and questions regarding: the use of surgical equipment; wound healing processes; and even anaesthesia (Naderi et al., 2004).

The importance of muscle and ligaments surrounding the spinal cord was identified in the 15th century by Leonardo Da Vinci. He suggested that the spinal cord actually functioned to give support to muscles and ligaments surrounding it, as well as give the body its anatomy (Sanan and Rengachary, 1996). During that time, anatomy textbooks were also published with spinal anatomy illustrations to show its parts and the dissections. Words like cervical, thoracic, lumbar, sacral, and coccygeal were also introduced and used in the textbook. A detailed textbook about spinal mechanics was published in 1680, by Giovanni Borelli (Sanan and Rengachary, 1996).

Later in the 19th century, the use of surgical instruments and trauma care techniques were widespread. Aseptic techniques, x-ray images were developed and sterilisation of equipment through various stages of the surgery became common in order to lower the risk of infection. Surgeons could now locate the injury precisely and diagnose the symptoms and predict the outcome of the injury to a particular area. Antiseptics were also used along with immediate reduction of the injury and stabilization (Hanigan and Sloffer, 2004).

Common spinal disorders such as spinal stenosis (narrowing of the spinal canal) and spondylolisthesis (vertebral spillage) were recognized and named in 1950s. Advanced techniques such as anterior cervical discectomy and fusion had been invented and had attracted interest from the general public. Surgical microscopes and lumbar fusion hardware were also researched (or used) at this period but the result was disastrous, thus the technique was abandoned. In the 1970s, CT scanning and MRI scanning machines improved imaging of the spinal column and spinal cord. During this period, surgical techniques for fusion were revised and

improved. Disc replacements had become a popular methodology along with synthetic bone and bone stimulators in the 1980s when the biology of the spine became the central focus (Perovitch et al., 1992, Pearce, 2008). Further development in fibre optics and high resolution cameras such as for laparoscopy, thorascoscopy and endoscopy also aided and led to the technique of 'visual assisted surgery' (VAS), 'minimally invasive surgery' (MIS), and 'minimal access surgery' (MAS).Treatment approaches such as the use of the steroid drug, Methylprednisolone, was also discovered in the 1990s and provided doctors with another treatment option (Lifshutz and Colohan, 2004). This drug was used in the clinical trials for years prior to optimising the most effective dosage for patients. It was found that methylprednisolone, an anti-inflammatory drug, produced the most effective result when administered in high doses within the first eight hours of an acute injury. This drug is known to decrease the damage caused by the inflammation of the lesion and the bursting of damaged cells (Bracken et al., 1984). At that time, the outcome of the treatment was not predictable, but doctors could now determine whether the treatment actually provided any benefit to the patient.

As spinal cord disorders are complex, a deeper understanding of their mechanism is required in order to assess an individual with a SCI. An effective therapeutic method is necessary to treat SCI to achieve a high degree of autonomy and a better quality of life for many years after the time of injury. New devices to reduce pain, advance techniques to aid the surgery and new treatments are being investigated and developed. Currently, treatment strategies that can restore spinal cord injury to the same level of function that existed prior to the injury are not yet available. This continuing research aims to reduce the neurologic deficits, improve functioning

in SCI patients and assist patients in readjusting to a suitable lifestyle that will allow them to engage in certain living activities. Along with traditional SCI treatment, recent innovative technologies and surgical procedures hope to offer the patient strategies to augment and mobilise the paralysed muscles and lead life independently.

1.2 CENTRAL NERVOUS SYSTEM INJURY

The central nervous system (CNS) is comprised of the brain, brain stem and spinal cord. It is considered to be one of the most complex and enigmatic systems in the body (Raineteau, 2008). The intricate network of the CNS is responsible for controlling behaviour, consciousness, sensation and movement. It contains billions of neurons which have axons that either interconnect with each other in specific ways or are connected via the peripheral nervous system (PNS) to muscles and other tissue in the body (Lagercrantz and Ringstedt, 2001, Schwab, 2002). However, these functions may be disrupted when there is a lesion to the CNS, causing a loss in communication between the brain and the other parts of the body. Often, many neuronal cell bodies survive after such a lesion but the axons become severed. Whilst the distal part of the severed axon disintegrates, the proximal part which remains attached to its parent cell body survives as long as its parent cell body does not undergo cell death (Horner and Gage, 2000).

Nervous system-related injuries consist of a diverse set of disorders. One major cause is neurotrauma which results from accident-related injuries such as Spinal Cord Injury (SCI) and Traumatic Brain Injury (TBI). The injury can lead to permanent severe disability depending on the region affected (LaPlaca et al., 2007). Other disorders are non-traumatic related, caused from various medical conditions such as

tumours, neurodegenerative diseases (Alzheimer's, Parkinson's and Huntington's disease) and neurovascular diseases which often lead to stroke. These conditions result in the loss of neurons and damage to axons, eventually lead to dysfunction of the CNS (Wootla et al., 2012). In mammals neurogenesis is largely restricted to early life with only very small numbers being generated in adult life, and often in only very specific brain regions. Neurons lost due to cell death in adult mammalian life, no matter what the cause, cannot be easily replaced (Schwab and Bartholdi, 1996, Schwab, 2002, Ramer et al., 2005, Maier and Schwab, 2006).

1.3 THE IMPACT OF CNS INJURY

Spinal cord injury is considered to be one of the major causes leading to a loss of mobility. Damage to the spinal cord often results in permanent disability or loss of movement (paralysis) and sensation below the site of the injury (Price et al., 1994, Rolls et al., 2008, Garbossa et al., 2012). Approximately, 10,000 people per year in the United States suffer from a disability related to spinal cord injury. There is no cure for this dreadful condition (Kurtzke, 1977, Rolls et al., 2008). The injury causes a devastating impact on every aspect of a patient's life including physical, emotional and social health (Bareyre, 2008, Vawda et al., 2012). Most of spinal cord injury occurs as a result of motor, sporting or industrial accidents (Molsa et al., 1999).

1.4 HUMAN SPINAL CORD

The adult human spinal cord is about 45-60cm (18-24 inches) long and extends from the base of the brain, down to the lower back. The brain and spinal cord constitute the CNS and the motor and sensory nerves outside the CNS make up PNS (Berman et al., 1998). The spinal cord is made up of grey and white matter. The grey matter is located in the middle of spinal cord surrounded by white matter, It is made up of neuronal cell bodies as well as four horns extending off from each corner whereas, the white matter contains myelinated axons which interconnect the various brain and spinal cord regions that carry these connections provide a 'relay' from brain to the periphery and vice versa. The spinal cord is divided into 31 segments, each with a pair of anterior (motor) and dorsal (sensory) spinal nerve roots. Anterior and dorsal roots combine to form the spinal nerve on each side of the body, which exits from vertebral column through foramina (Goldstein, 2002).

The activity of the spinal cord is regulated by the two types of motor neurons known as upper motor neurons and lower motor neurons (Whiting, 1948). Upper motor neurons are located in the cerebral cortex and basal nuclei of the brain and their main function is to carry motor information down to the final pathway (lower motor neurons). The lower motor neurons are found in the anterior horns of spinal cord grey matter. They connect the spinal cord to other parts of the body and are responsible for sending messages from the brain to the body parts in order to stimulate actions such as muscle movement (Chen et al., 2004, Gordon et al., 2009, Van den Berg-Vos et al., 2009, Garbossa et al., 2012).

The spinal cord is well-protected and surrounded by rings of bone called vertebrae which constitutes the spinal column. The spinal column is made up of 33 bones called vertebrae, each with a circular opening. The bones are stacked one on top of the other and the spinal cord runs through the hollow channel in the stacked bones. The vertebrae are divided into sections and are named and numbered from top to bottom according to their location along the backbone (Sherman et al., 1990, Ogawa et al., 2008):

- Cervical vertebrae (1-7) are located in the neck, thoracic vertebrae (1-12) in the upper back which is attached to ribcage
- Lumbar vertebrae (1-5) in the lower back
- Sacral vertebrae (1-5) in the hip area
- Cocygeal vertebrae (1-4) in the tailbone.

The spinal cord contains nerve cells, surrounded by long tracts of nerve fibers consisting of axons. The tracts extend up and down the spinal cord, passing signals to and from the brain. When these bones are displaced or broken, the axons can be compressed or severed, resulting in a disruption of communication between the brain and other parts of the body. Thus, messages can no longer be transmitted across the injured area (Liu et al., 1999).

The effects of SCI depend on the type, level and severity of the injury. The injuries can be classified as complete or incomplete transection depending on whether there is a total or partial loss of sensation and movement below the level of injury (Al-Habib et al., 2009). A complete injury can be defined as where there is loss of motor

and sensory function below the level of injury and both sides of the body are equally affected. An incomplete injury is characterised by the fact that there are some sensations or movements retained below the level of injury (Valles and Mearin, 2009). An injury at the cervical region, with associated loss of muscle movement of both arms and legs, causes quadriplegia. An injury in the lower part of the spine such as in thoracic or abdominal region only affects the lower part of the body including the legs, resulting in paraplegia (Krassioukov, 2009).

Currently, there is no cure for spinal cord injury. Short-term treatments with drugs to reduce secondary damage due to inflammation are available, yet there is no effective treatment for SCI because the axons are unable to regrow after injury (Tederko et al., 2009, Vawda et al., 2012).

1.5 AXONAL REGENERATION AND DENDRITIC GROWTH

A central problem in neurodevelopment is determining morphologically and functionally distinct axons and dendrites. Neurons are divided into three parts: dendrites, axons and cell body (soma). Dendrites are branches protruding out of a neuron. They receive electrical contacts from other neurons either by axo-dendritic or dendro-dendritc synapses Different types of neurons can have a morphologically distinct pattern of dendritic branching that can influence both the numbers and types of their synaptic interconnections with other neurons. Axons, on the other hand are the “output” point of neurons. They are long fibers of a nerve cell that conduct electrical impulses away from the neurons ‘soma. In the CNS axons generally make synaptic connect with the dendrites or cell bodies of their particular target neuron. In the PNS they can make special types of connections to the muscles which they

control. Axons are often “wrapped” with a layer called myelin sheath which contributes to faster movement of messages to the brain. It has been suggested that proteins (microtubules, microfilament and actin) play a crucial metabolic activity within the axons. Thus, when axons are damaged, the metabolic synthesis of protein in the cell body is dys-regulated and regeneration fails to take place. Dendrites can also be distinguished from axons in the pattern of growth, development and morphology. During development, one cell process of the postmitotic neuron is identified as the axon, and the remaining processes are specified as dendrites. Neurons only ever have one axon although they can have any number of dendrites. One main difference that appears during maturation of axon and dendrites is their intrinsic property in terms of microtubule polarity. A study by Bass (1988) used an electron microscope to demonstrate that axons of mature neurons in vertebrate species are composed of microtubule that were oriented exclusively with the plus-end projecting away from the soma, whereas, the dendrites were composed of a variety of microtubules with mixed polarity. The pattern of microtubule organization and polarization can be assessed in neuronal development in vitro to differentiate axons from dendrites (Baas et al., 1988). A study by Kollins (2009) demonstrated that dendrites contained more immature, dynamic microtubules throughout the arbor than the axons. Microtubules cytoskeleton and intercellular membranes play a vital role in transporting molecules for extending dendritic networks. These differential organization patterns between dendrites and axons are likely due to separate dendrites and axon development (Kollins et al., 2009). Thus, further studies on how microtubules are organized during development may reveal the mechanisms of neuron maturation, dendrite plasticity and regeneration of axons following injury.

Injuries to mammalian peripheral nervous system (PNS) have shown some capacity for spontaneous repair as the axons within PNS have the ability to regenerate and form new connections with target areas, thereby restoring some functions (Qiu et al., 2000, Huebner and Strittmatter, 2009). In contrast, axons in the mammalian central nervous system (CNS) have a limited capacity for the spontaneous regrowth after injury. It is thought that this is due to the fact that the environment provided by the post injury CNS contains many factors that inhibit axonal regeneration (Huebner and Strittmatter, 2009, Nishio, 2009, Tom et al., 2009).

In contrast to mammals, it has been found that some sub-mammalian species such as amphibians and teleost fish are capable of repairing injuries to their CNS (Skene, 1984, Schwab and Bartholdi, 1996). Zebrafish seem to contain few, if any, inhibitory factors in their CNS (Sivron and Schwartz, 1995). Questions arise regarding the evolutionary processes that have led to a differentiation between mammals and these sub-mammalian species. Sub-mammalian species (reptiles and amphibians) are capable of repairing injuries to their CNS, whilst mammalian species are unable to repair such injuries. It is not yet clear how these sub-mammalian species regenerate new neurons as well as repair the damaged ones after axonal transection. A deeper understanding of the differences between these species may give insight on how to repair the injured mammalian CNS (Coggeshall and Youngblood, 1983, Bareyre, 2008).

The long established assumption that axons in the mammalian CNS are inherently incapable of regeneration has been recognised as no longer acceptable. A number of studies have clearly shown that under certain conditions, adult CNS neurons are able to regenerate and repair after injury (Ramón y Cajal, 1928, Benfey and Aguayo,

1982). Proper growth and arborisation of dendrites/ axons are crucial for proper functioning of the nervous system. This project aims to examine the mechanisms of axonal regeneration in adult zebrafish after injury. It has been suggested that axonal regeneration fails in post-lesion mammalian CNS because of inhibitory factors that make the CNS environment unable to support such growth (Liu et al., 2008).

Many studies have demonstrated that axonal regeneration is limited by inhibitory influences present in the injured CNS environment that preclude the regrowth of injured axons. These include inhibitory molecules and 'reactive astrocytes' that make the CNS environment non-permissive for axonal growth (Reier and Houle, 1988). In addition, it has been shown that various neurotrophic factors are required to promote successful axon regeneration and path-finding in the CNS (Liu et al., 2008).

1.6 TREATMENT STRATEGIES FOR SPINAL CORD INJURY

Various strategies and approaches have been used to treat spinal cord injuries. Currently, there are several approaches being investigated including both *in vitro* and *in vivo* testing using animal models (Bunge and Pearse, 2003, Bramlett and Dietrich, 2007, Dasari et al., 2007, Bunge, 2008). The strategies that are being examined include targeting cell signalling factors and grafting of peripheral nerves to the lesion (Rossignol et al., 2007). Although there have been some positive results, more effective treatment methods are still required to improve motor function after spinal cord injury (Pearse and Barakat, 2006, Cardenas and Felix, 2009, Vawda et al., 2012).

1.7 REGENERATION IN THE ADULT MAMMALIAN NERVOUS SYSTEM

1.7a) Regeneration in the peripheral nervous system (PNS)

The most prominent characteristic that distinguishes the PNS from the CNS is its capacity for axonal regeneration (Gaudet et al., 2011). The axons in the PNS are surrounded by Schwann cells whereas in the CNS, myelin sheaths are formed by oligodendrocytes (Arroyo and Scherer, 2000, Baumann and Pham-Dinh, 2001, Quarles, 2005). In the PNS, regenerating axons emerge from the proximal stump of the lesion growing into the distal nerve and can extend to the target regions. It has been long known that axons within mammalian peripheral nerves that have been injured or severed can show some capacity for regeneration and remyelination (Ann et al., 1994, Ide, 1996, Ferguson and Son, 2011).

1.7b) Wallerian Degeneration

Severing the peripheral nerves causes disruption of the axons. The portion of an axon proximal to a lesion (proximal stump) remains attached to its parent cell body whilst the segment distal to the lesion (distal stump) becomes separated. On occasion, the cell body of an injured axon may die as a result of an injury. Such neurons normally cannot be replaced. Severed axons whose parent cell bodies have not died as a result of an injury usually degenerate as far back as the first internode (Stoll and Muller, 1999, Johnson et al., 2005). At the same time, the cell body begins to up-regulate certain genes, including immediate early genes, in preparation for axon regeneration (Gillingwater and Ribchester, 2001, Gaudet et al., 2011). The

distal segment of the axon with its myelin sheath also disintegrates, and the debris is phagocytosed by invading macrophages (Stoll and Jander, 1999, Johnson et al., 2005). Schwann cells, which carry out peripheral axon myelination, proliferate and are thought to upregulate various neurotrophic factors to prepare the distal segment of the nerve for regenerating axons (Ide, 1996). These grow from the proximal segment of the nerve into the neurolemmal bands of Büngner that are left behind after the degeneration of the original axons (Fernandez-Valle et al., 1995). These bands of Büngner are thought to both aid the regeneration process and may also be involved in guiding the regenerating axons to their correct target tissues. Sprouting axons from the proximal stump have been shown to be capable of growing into the distal stump segment. These newly regenerating fibres may enter the band of Büngner and continue to regrow at the rate of 1-2mm a day. Further Schwann cell division takes place and these cells aid in the remyelination of the newly regenerated axons (Tetzlaff, 1982). This process of degeneration of injured axons is termed Wallerian degeneration (Stoll and Muller, 1999). Research by Ide (1983) has shown that the presence of Schwann cells is of crucial importance to the process of axonal regeneration. This has been shown in experiments where Schwann cells within segments of peripheral nerve grafts were killed by repeated freezing and thawing procedures. Such nerve segments could not support axonal regeneration until Schwann cells were re-introduced into the grafts (Ide, 1983).

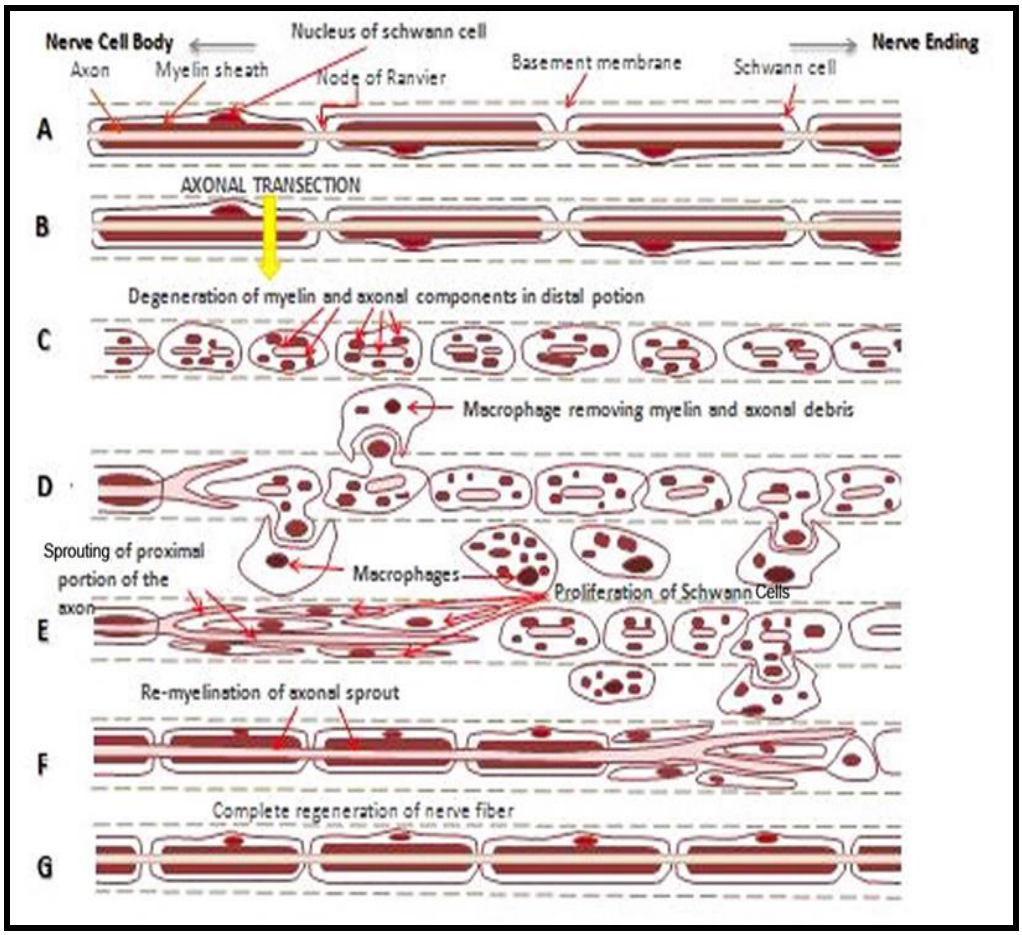

Nerve Cell Body
Nucleus of schwann cell
Nerve Ending
Axon
Myelin sheath
Node of Ranvier
Basement membrane
Schwann cell
A
AXONAL TRANSECTION
B
Degeneration of myelin and axonal components in distal potion
C
Macrophage removing myelin and axonal debris
D
Sprouting of proximal portion of the axon
Macrophages
Proliferation of Schwann Cells
E
Re-myelination of axonal sprout
F
Complete regeneration of nerve fiber
G

Figure 1.1: Wallerian Degeneration.

Normal appearance of axon with the node of Ranvier and, b) Transection of the neurons resulting in the distal fragmentation of the axon and myelin. c) The proximal stump retrograde degeneration occurs back to the first internode. d) Neuronal cell body is swelling and Schwann cells proliferate in the distal stump. Macrophages and Schwann cells phagocytose axonal debris and e) Schwann cells in the distal stump form band of Bünger and sprouting is occurring from the proximal stump. f) and g) This is followed by a complete regeneration of peripheral nerve axons. *Adapted from* (Lundborg, 1988)

1.8 REGENERATION IN THE CENTRAL NERVOUS SYSTEM (CNS)

It has generally been believed that neurons in the mammalian CNS fail to regenerate after injury. Trauma in the CNS leads to severe damage and persistent functional deficits (Yang and Yang, 2012). However, evidence from many recent studies suggests that CNS neurons are capable of regenerating their axons if provided with the appropriate environmental conditions (Cui, 2006, Rossignol et al., 2007, Tom et al., 2009). It is now clear that the failure of axonal regeneration in the adult CNS neurons is not due to the lack of an intrinsic capability to regrow axons, but mainly attributed to the presence of inhibitory factors in the post-lesion CNS environment along with the formation of glial scar tissue (Neumann and Woolf, 1999, Lu et al., 2007). Experimental approaches facilitating axonal regeneration in mammalian CNS are continuing and successful regeneration may be achieved through one or more of the following strategies such as

a) Alteration of the environment to favour regeneration in the CNS such as providing peripheral nerve bridges, or the transplantation of Schwann cells.
b) Application of neurotrophic factors to the cell body and axon tips.
c) Blockage of growth-inhibitory molecules such as myelin-associated glycoprotein, myelin oligodendrocyte glycoprotein and Nogo-A.
d) Prevention of chondroitin sulphate proteoglycans related scar tissue formation at the lesion site using chondroitinase ABC.
e) Elevation of the intrinsic growth of injured axons by increasing intracellular cyclic adenosine monophosphate levels (CAMP).

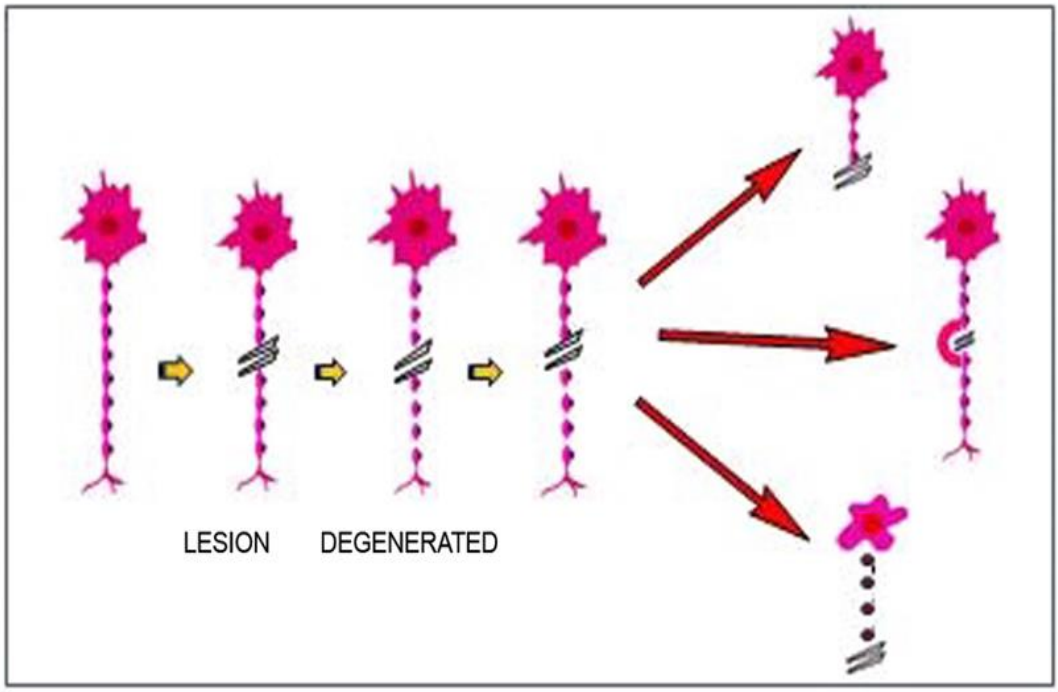
The approaches which yield a positive outcome may potentially be used to enhance the regeneration of injured CNS axons. This regeneration includes keeping neurons alive in the growth state and preventing the formation of glial scar (Cui, 2006).

1.9 POST-INJURY CHANGES TO THE CNS ENVIRONMENT

Injuries in the mammalian CNS may cause a complex sequence of pathological responses (Hall and Braughler, 1993, LaPlaca et al., 2007). Immediately after injury, there are extravasations of blood into the lesion site due to the disruption of the blood vessels resulting in local ischemia, hypoxemia and hypoglycaemia (Schwab and Bartholdi, 1996, Dumont et al., 2001). The primary mechanical damage is followed by a complex process of secondary damage in which ischaemic and inflammatory processes play a major role. These actions cause the activation of microglia and macrophage invasion at the site, this in turn contributes to the scar formation (Stichel and Muller, 1998). Scar formation by astrocytes and microglia is a result of the body re-establishing the blood brain barrier, thus preventing further injury to the CNS. When the axonal growth is terminated, astrocytes which play a role in providing substratum for migratory neurons and growing axons during CNS development seem to lose their ability to support axon growth. The astrocytes become “reactive” and actively block axonal growth distal to the lesions. Recently, reactive astrocytes within the glial scar have been shown to up-regulate molecules such as tenascin, semaphorin 3, slit proteins and chondroitin sulphate proteoglycans (Brodkey et al., 1995, Apostolova et al., 2006). It has been demonstrated that the molecular composition of the scar and the production of inhibitory molecules by astrocytes contributes to regenerative failure (Qiu et al., 2000).

Secondary damage causes the local loss of neuronal elements, myelin damage and vascular changes. Several weeks after the injury, macrophages clear the tissue debris at the lesion site, resulting in cyst formation and cavitation which characterises the patho-physiological evolution of spinal cord injuries (Bregman, 1998, Liu et al., 2006). It has been demonstrated that although, the majority of CNS neurons die after disconnecting from target areas, some are able to survive and regrow their axons within the lesion sites. Adult dorsal root ganglion neurons were tested and micro-transplanted into undamaged white matter tracts in the CNS. This micro-transplantation technique prevents an inflammatory response of neurons and can immediately access the host environment .The axons then regenerate over long distances within the white matter tracts in the CNS (Davies et al., 1993, Davies et al., 1997, Houweling et al., 1998). However, when axons reach the injured site, the newly formed growth cones become exposed to inflammatory infiltrates and inhibitory extracellular matrix molecules. Subsequently, the growth cones converted into a dystrophic state and were unable to continue long distance regeneration. The sprouting tips of severed axons also remained in the same place without further growth. The collapsing of growth cones are thought to be caused by the non-permissive nature and the presence of inhibitory molecules in the CNS (Horn et al., 2008, Kigerl et al., 2009).

A


LESION
DEGENERATED

B

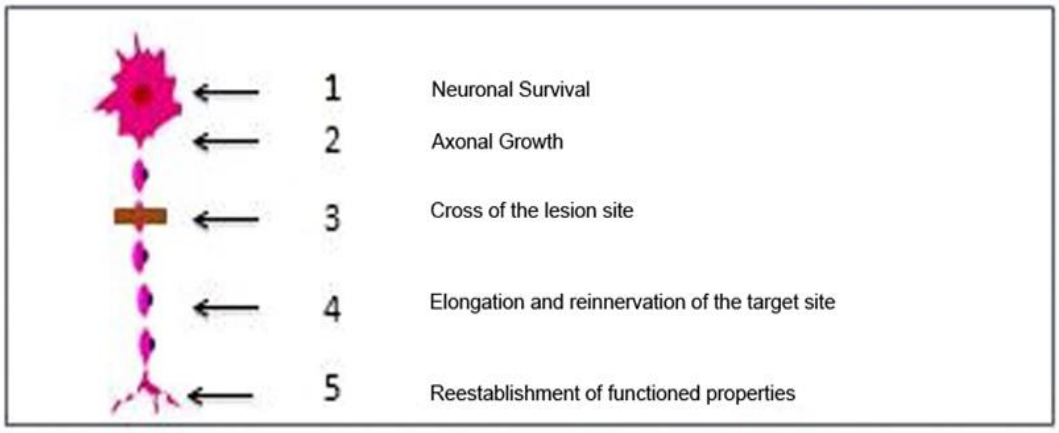

1
Neuronal Survival
2
Axonal Growth
3
Cross of the lesion site
4
Elongation and reinnervation of the target site
5
Reestablishment of functioned properties

Figure 1.2: Injury to CNS neuron.

a) Schematic summary of the cascade of cellular reactions necessary to restore the CNS functions after axonal disruption in five phases. Pathological changes of the lesion axons in mammalian CNS. The lesion causes axonal disruption and undergoes degeneration. The axon degenerates in the anterograde and retrograde direction. Subsequently, the axon sprouts up to the lesion site spontaneously and eventually stops growing abruptly and never succeeds in crossing over the site but persists at the border of the lesion area. B) This diagram illustrates the requirement for structural and functional repair of the lesioned axons and re-establishment of functional properties. The axon and the surrounding myelin break down during the injury. The axon then starts to regenerate and re-establishes new connections and function is eventually restored. *Adapted from* (Stichel and Muller, 1998)

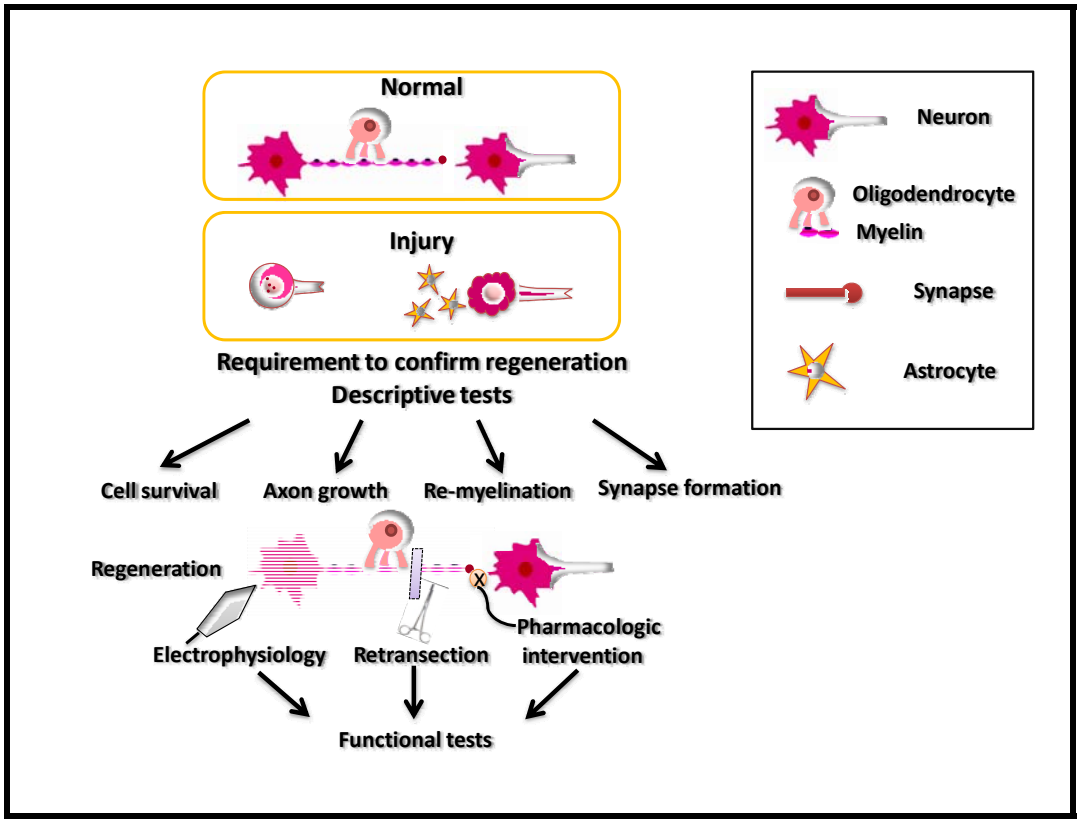

Normal
Injury
Requirement to confirm regeneration
Descriptive tests
Cell survival
Axon growth
Re-myelination
Synapse formation
Regeneration
Electrophysiology
Retransection
Pharmacologic intervention
Functional tests
Neuron
Oligodendrocyte
Myelin
Synapse
Astrocyte

Figure 1.3: A process of axonal regeneration after lesion in CNS.

This diagram shows several important multiple steps for axonal regeneration to take place after injury. Initially, the lesion axon must survive and progress to the original target site. Once the contact is connected, the axons will undergo the remyelination process and eventually form appropriate synaptic connection for restoring functions. Electrophysiology and pharmacologic intervention can be further used to determine the function and specificity of the generated pathway of axons. *Adapted from* (Ma et al., 2011)

1.10 CNS NEURONS CAN REGENERATE FOLLOWING INJURY- AGUAYO'S EXPERIMENT

Previously, it was not clear whether CNS neurons lack the capacity for axonal regeneration after development or if the environment became non-permissive for regeneration. In a series of studies by Aguayo's group, it was shown that wide varieties of CNS neurons were able to regenerate if they were allowed to grow in peripheral nerve grafts. In the experiments, PNS grafts were introduced into the cerebral hemisphere where they were allowed to form a bridge between the medullar and thoracic spinal cord. (Aguayo et al., 1981, Benfey and Aguayo, 1982). It was found that the severed central axons extended up to 35mm into the transplanted nerve graft in comparison to the short distance of less than 1 mm that regenerating central axons typically grow within the CNS (Benfey and Aguayo, 1982). However, growth ceased once the regenerating axons met the graft-CNS interface at the opposite end of the graft. These results strongly supported the hypothesis that major differences between CNS and PNS micro-environments are limiting factors in axonal regeneration, not the intrinsic properties of the neurons themselves. This evidence has placed more prominence on the importance of elucidating the role of inhibitory substances within the post lesion CNS environment.

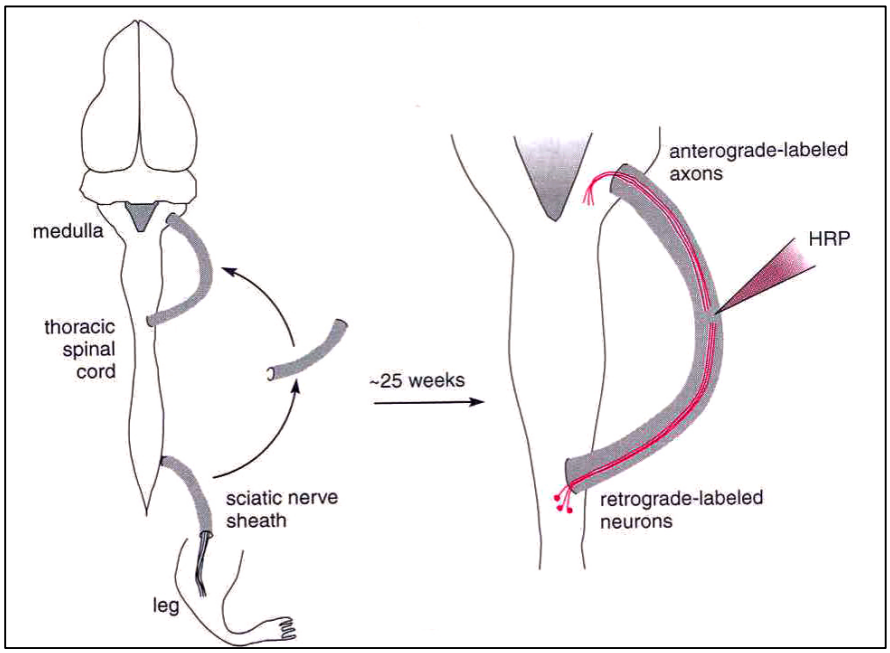

medulla
thoracic spinal cord
sciatic nerve sheath
leg
~25 weeks
anterograde-labeled axons
HRP
retrograde-labeled neurons

Figure 1.4: Peripheral Nerve Transplant.

A peripheral nerve “bridge” can support the growth of axons from neurons located within the CNS. In these experiments, a short piece of sciatic nerve was transplanted to form a bridge between the medulla and thoracic spinal cord (left figure).The axons originally within the transplanted segment die, leaving non-neuronal tissue. After a period of survival, HRP label was injected into the nerve segment (right figure). Neuronal cell bodies located within the medulla and thoracic spinal cord were found to become labelled with HRP showing that they had extended axons into the transplanted nerve segment. *Taken from* (Sanes et al., 2000) *modified from* (Aguayo et al., 1981)

1.11 STEM CELLS

The possible use of stem cells to repair the injured nervous system offers another possible therapeutic approach to repair the injured CNS. It has been thought that multipotent neural stem cells could enhance neural repair after spinal cord injury either by replacing host cells that have died or by facilitating host axonal growth. Neural Stem Cells (NSCs) are undifferentiated nervous system cells that are capable of proliferation, repeated subculture (self-replicating capacity), and differentiation into the three main types of cells comprising the central nervous system, that is, neurons, astrocytes, and oligodendrocytes (multipotency) (Cao et al., 2002). Studies are in progress throughout the world examining two major areas of research to develop therapeutic strategies for CNS injuries using NSCs:

(i) The activation of endogenous NSCs.
(ii) The transplantation of NSCs.

It is possible that stem cells, perhaps, in combination with various neurotrophic factors could promote the CNS repair by reconstituting a “bridge” through a lesion site to aid axonal regeneration (Magnus and Rao, 2005). Some stem cells are pluripotent *in vitro* as they can form both neurons and glia. However, it has been found that when the cells are injected into a specific CNS site, they can become restricted to a given cell lineage (Bottai et al., 2003). Thus, stem cells injected into the spinal cord can become restricted to glial lineages. Stem cells, when transplanted into the hippocampus, have been shown to take on neuronal as well as glial phenotypes. These findings suggest that the spinal cord is an environment that favours glial but not neuronal differentiation. Another possibility is that stem cells

could be used to help promote remyelination and consequential functional recovery. Yet, the mechanisms underlying such trophic effects have not been fully explored or defined (Garbuzova-Davis et al., 2006).

1.12 INHIBITORY MOLECULES ASSOCIATED WITH AXONAL GUIDANCE DURING DEVELOPMENT

As mentioned earlier, following injury to the adult mammalian CNS, regenerative growth of severed axons is extremely limited (Colamarino and Tessier-Lavigne, 1995). The failure of regeneration in the CNS is due to the presence of molecules capable of inhibiting or blocking growing axons. Therefore, the molecular mechanisms associated with axonal guidance during development and their roles in regeneration of the spinal cord have been studied (Schwab and Bartholdi, 1996).

1.13 MECHANISM OF AXON REGENERATION

During the embryonic stage of development, the extension and elongation of axons occurs by a particular mechanism. This includes exploring the environment and reorganising the cytoskeleton in the suitable direction of growth (Bentley and O'Connor, 1994, Dent and Gertler, 2003). The growth cone has a broad flat proximal portion. The lamellipodium gives off numerous thin figure-like projections, called filopodia, to scan the environment (Bentley and O'Connor, 1994). These filopodia allow the axons to respond to the external cues that guide them to the target (Selzer, 2003).

During this development process, the axon contains microtubules and actin filaments but not neurofilament protein. Filamentous actin (F-actin) is highly concentrated at

the growth cone (Jones et al., 2006). At the filopodium surface, transmembrane proteins such as integrins act as receptors for cell adhesion molecules. The adhesion to an appropriate substrate activates a transmembrane signal that causes F-actin polymerisation resulting in elongation of the filopodium (Lin and Forscher, 1993). The growth cone is also composed of myosin, which links the actin microfilaments in the filopodia to the microtubule system in the axon (Selzer, 2003, Zhang et al., 2005a). F-actin then binds to myosin and generates a force, pushing the axon to extend in the direction of the filopodium, resulting in a high growth rate (1-5mm/day) (Bentley and O'Connor, 1994).

Even though axonal regeneration in the CNS is assumed to use this mechanism, the assumption has not been clearly proven. Various studies (Yamashita, 2007, Giger et al., 2008, Ma et al., 2011) have demonstrated that the growth of mature axons after lesion may involve different mechanisms (Selzer, 2003).

1.14 SPECIFICITY OF REGENERATION

Studies using the optic nerves of fish and frogs have indicated that the mature CNS retains the ability to guide regenerating axons along the correct path (Grafstein, 2006). This specificity is due to repulsive and attractive interaction between molecules and ligands that are expressed in the neurons (Liu et al., 2009). A wide variety of CNS inhibitory molecules that act as attractive or repulsive cues in the developing nervous system have been identified. These include the myelin inhibitors (a molecule in the myelin that inhibits myelination) such as Nogo-A, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp) and several types of chondroitin sulphate proteoglycans (CSPGs) (McKeon et al., 1995,

Filbin, 2003, Schwab, 2004, Liu et al., 2006).There is evidence showing that axon guidance molecules belonging to semaphorin 3A, ephrin, and netrin families contribute to the hostile environment of injured CNS tissue (Benson et al., 2005, Low et al., 2008).

1.15 CELL ADHESION MOLECULES OF THE IMMUNOGLOBULIN SUPERFAMILY IN AXONAL REGENERATION AND NEURAL REPAIR

Intracellular adhesive interactions play a pivotal role in both mature and developing nervous systems (Crossin and Krushel, 2000, Kiryushko et al., 2007). During the development stages, cell adhesion molecules (CAM) are involved in cell migration; axon guidance; target recognition and synapse formation and modulation. However, observations in mature nervous systems suggest that cell adhesion molecules tend to stabilize synaptic connection, cell-cell contacts and neurons-glial interactions (Sakisaka and Takai, 2005). Injuries to the CNS inhibit the stable state of tissue resulting in the death of neurons. This then leads to CAMs participating in repairing the damaged tissues, reconstructing the lesion site and regenerating the axons (Zhang et al., 2008).

CAMs are glycoproteins that are found on the cell surface and mediate cell-cell extracellular matrix (ECM) (Grumet, 1991, Takeichi, 2007). The cell adhesion molecules that are expressed in the CNS can be divided into three classes based on their sequence structures: integrins, cadherins and members of the immunoglobulin (Ig) family (Fercakova, 2001). It has now been demonstrated that CAMs act not only as regulators in cell adhesion, but also act as signal transducing receptors (Zhang et al., 2008).

The Ig superfamily can be sub-classified into at least five groups of related molecules based on the number of Ig-like domain such as fibronectin type III, neural cell adhesion (NCAM), L-1, TAG-1, and myelin associated glycoprotein (MA) (Grumet, 1991, Takeichi, 2007). However, most studies have focused on the involvement of neural cell adhesion molecules (NCAM) and L-1 on axon regeneration after nerve injury and their potential application in the treatment of CNS injury (Zhang et al., 2008).

1.15a) L-1 CAM

L-1 CAM belongs to the CAM subfamily which includes neurofascin, NrCAM, ABGP and CHL1, a close homolog of L1 (Chaisuksunt et al., 2000). L-1 CAM mediates cell adhesion by hemophilic interactions and also participates in binding hetrophilic ligands such as TAG, NCAM, and proteoglycans (Lemmon et al., 1989). The L-1 molecule is highly expressed in the developing nervous system and facilitates cell migration, axonal guidance and axonal fasciculation (Coman et al., 2005, Ditlevsen et al., 2008).

During the development of peripheral nerves, L-1 is localised on small, non-myelinated axons (Mirsky et al., 1986, Martini, 1994). In the initial phase of myelination, when Schwann cell processes have completed approximately 2 loops around the axons, L-1 is no longer detected. This finding suggested that L-1 is involved in the initiation of axonal-Schwann cell interaction and the onset of myelination (Martini and Schachner, 1986).

The application of L-1 has been used for neural repair as it has the ability to transduce signals into neurons and participates in the regenerating process by

providing growing axons with permissive substrates (Silver and Miller, 2004). L-1 can be administered *in vivo* by direct injection of viral vectors carrying the L-1 gene around the injury site. It is also known that over-expression of this molecule causes a decrease in the expression of NG2 (a CSPG which inhibits neurite outgrowth *in vivo*) (Chen et al., 2007).

1.15b) N-CAM

N-CAM belongs to the Ig family of CAMs, and is derived from a single gene but is expressed in several isoforms that result from the alternative splicing of mRNA (180kD, 140kD, 120kD) (Gennarini et al., 1986, Murray et al., 1986). The three isoforms of NCAM have five Ig loops followed by a FNIII domain. All N-CAMs participate in both homophilic and hetrophilic interactions with the neighboring cells (Soroka et al., 2003, Walmod et al., 2004). However, these three isoforms show different expressions relative to cell types. NCAM 180 is predominantly expressed by neurons in late development. NCAM 140 is expressed in glial cells and developing neurons locating to growth cones. NCAM 120 is restricted only to glial cells (Cunningham et al., 1983, He et al., 1987, Buttner et al., 2003).

NCAM carries a carbohydrate moiety such as polysialic acid (PSA). PSA is a linear polymer of N-acetylneuraminic acid in α-(2-8) linkage (Finne et al., 1983). NCAM-PSA is expressed by all immature neurons, indicating that PSA is crucial for NCAM function in the development of neural tissues. The expression of both NCAM and PSA-NCAM is developmentally regulated. However, the regulation of PSA is independent of the expression of NCAM (Zhang et al., 2008).

Polysialyation may also occur in glial cells such as astrocytes. Most of the astrocytes do not express PSA. However, after CNS injury, PSA is expressed in astrocytes in the area of reactive gliosis. It has also been reported that the over-expressing of PSA (sialic acid component) associated with NCAM protein could possibly enhance the plasticity of regenerating axons and may provide a permissive environment for neurite outgrowth (El Maarouf et al., 2006).

Given the role of PSA-NCAM in migration of neurons and the guidance and targeting of axons, it is proposed that engineered expression of PSA might be able to enhance axon growth after the injury. One approach is to inject polysialyic transferase to the lesion site using a viral vector. It has been shown that corticospinal tract axons proximal to the injury site can grow across to the distal spinal cord by such treatment (Zhang et al., 2008). These axons are able to grow beyond the PSA-rich region, suggesting that PSA can render the scar permissive to the sprouting axons. Application of antibodies against PSA results in path-finding errors in the axons of retinal ganglion cells (RGCs). This suggests that the action of PSA on axons may regulate growth cones in response to environmental cues (Zhang et al., 2007).

In CNS, PSA-NCAM is associated with axonal elongation mainly on the axonal growth and filopodia. This indicates that the hemophilic adhesive action of NCAM may decrease during regeneration. Remarkably, PSA-NCAM is one of the markers for regenerating axons in the CNS (Zhang et al., 2008).

The poor recovery of the CNS following an injury usually results in a glial scar formation around the injury site. This glial scar consists of reactive astrocytes, a mixture of growth-promoting molecules and growth factors combined with growth

inhibitory molecules (Faulkner et al., 2004). Unfortunately, inhibitory molecules dominate in the CNS environment, resulting in axon growth inhibition (Rolls et al., 2009). The reactive astrocytes act as a physical and chemical barrier by releasing inhibitory factors such as chondroitin sulphate proteoglycans (CSPG). CSPGs families are known to have a significant role in blocking the axon growth by interfering with integrin signaling. A clear understanding of how signaling helps to promote growth factor-induced axon growth would provide methods to enhance axon regeneration over the inhibitory molecules (Bradbury et al., 2002).

Although the mechanisms that inhibit the regeneration in CNS are not fully known, there is enough evidence to support the suggestion that the molecular composition of extracellular matrix (ECM) is another factor regulating and contributing to axonal regeneration (Gu and Lu, 2007). ECM molecules like laminin, fibronectin, proteoglycans, collagen, etc. play a crucial role in neurite outgrowth. For instance, laminin is a promoter of neurite outgrowth whereas proteoglycans inhibits them. In addition, hyalectans of CSPGs found in ECM of the CNS binds to both hyaluronan (HA) and tenascins and aid in supporting the structure of ECM (Crespo et al., 2007, Lin et al., 2008). Subsequently, CSPGs are intensively secreted following CNS injury and are shown to impede the axonal regeneration (Kwok et al., 2008).

The study by Rolls (2008) has demonstrated that transplanted DRG neurons can regrow their axons along the undamaged and degenerating site. It is now widely accepted that CSPGs can be broken down using the bacterial enzyme chondroitinase ABC (chABC). This enzyme acts by cleaving the GAG side chains on the CSPGs. However, chABC does not fully digest the GAG chains leaving behind side chains which are less inhibitory than the entire GAG chains, thus reducing the

capacity of CSPG's to act as inhibitors of axonal regeneration (Kilcoyne et al., 2012). chABC may also prevent formation of fibrotic scar in the axon tracts contributing to functional recovery in the CNS (Massey et al., 2006). Current research now focuses on the improved axonal regeneration by targeting the specific enzyme Xylosyltransferease-1. This enzyme acts on m-RNA causing GAG formation on the CSPGs (Shields et al., 2008). It is suggested that administration of this enzyme could possibly reduce the expression of GAGs on CSPG protein on both astrocytes *in vitro* and in the portion of tissue undergoing reactive gliosis after injury in *vivo*

1.16 AXON GUIDANCE MOLECULES

Many studies have produced outcomes that are consistent with the theory that CNS neuronal axons have the capacity to regenerate if provided with growth permissive environment (Aguayo et al., 1981, Benfey and Aguayo, 1982). Yet, it is still uncertain whether sprouting axons can bridge a lesion site. For this reason, guidance channels in the form of synthetic tubes commonly used in peripheral nerve repair are also being employed in the spinal cord injuries. Guidance channels allow regulatory factors and the cells from the distal stump to remain in direct contact with the site where the regeneration occurs (Kerschensteiner et al., 2005). Moreover, it also gives a direction to the axons crossing the gap and helps to separate the injury site from exogenous cells and tissues. Importantly, it prevents regenerating neurons from being exposed to astrocytes and various other inhibitory factors (Tuttle and O'Leary, 1998).

There are different types of tubes that have been used to improve axonal regeneration in the CNS. At this stage it seems that collagen tubes may be the

preferred type. Collagen is non-toxic, flexible, biodegradable, and permeable, thus, promoting regeneration. Furthermore, it has been shown to reduce glial inflammation that is formed after the injury (Shiga and Oppenheim, 1991, De Winter et al., 2002).

A study by Tsai (2004) has also suggested that these tubes have been shown to facilitate the regeneration of adult rat brainstem motor axons after spinal cord transaction. It was clearly demonstrated that axons from brainstem motor nuclei regenerated remarkably well through unfilled (no drugs or neurotrophic factors were present) synthetic hydrogel guidance channels and were able to bridge a 4mm gap between the stumps of a transected spinal cord of an adult rat (Tsai et al., 2004)The potential for greater recovery that may achieved by combining the multiple strategies such as the grafting of nerve section to the lesion, the application of neurotrophic factors, entubulation and stem cells approach (Oudega et al., 2001) has also been recognised. Such combinations may hold ~~a~~ promise as novel therapeutic strategies for treatment of CNS injuries.

1.17 NEUROTROPHIC FACTORS (NTFS)

Neurotrophins (NTRs) are a family of polypeptide growth factors that play a role in proliferation and differentiation, and act as a survival factors for many neuronal and non-neuronal cells (McAllister et al., 1999, Huang and Reichardt, 2001). In addition, these peptides are derived from a common ancestral gene and are similar in sequence and structure (Twiss et al., 2006). The family of neurotrophins includes Nerve Growth Factor (NGF), brain derived neurotrophic factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin-4/5 (NT-4/5), Neurotrophin-6 (NT-6) and Neurotrophin-7 (NT-7) (Levi-Montalcini, 1987, Bothwell, 1995, Lewin and Barde,

1996, Nilsson et al., 1998). The first four neurotrophins are best understood and most widely expressed in the nervous system. The NT-6 and NT-7 genes that encode neurotrophins have been found only in fish (Gotz et al., 1994, Nilsson et al., 1998). The effects of neurotrophins depend on the level of their availability, their affinity in binding to transmembrane receptors and the downstream signalling pathways that are activated after receptor activation. The neurotrophins are synthesised as precursors that are glycosylated and cleaved by convertases to release mature active proteins (12-14 kDa in size) (Chao and Bothwell, 2002). Although these proteins are commonly formed as non-covalently associated homodimers, some neurotrophin subunits are able to form heterodimers with other neurotrophins subunits. However, this physiological interaction remains unclear (Radziejewski and Robinson, 1993).

Neurotrophins exert their cellular effects through the action of two different receptors, the Trk receptor tyrosine kinase and the p75 neurotrophin receptors (p75 NTR), a member of the tumour necrosis factor (TNF) receptor superfamily (van der Geer et al., 1994, Roux and Barker, 2002). The Trk receptor comprises of an extracellular ligand-binding region, a single transmembrane domain and a highly conserved intracellular tyrosine kinase domain, while the p75 receptors consists of an extracellular ligand-binding region, a single transmembrane domain and an intracellular segment including a protein-association region known as the death domain (Huang and Reichardt, 2001, Hempstead, 2006, Reichardt, 2006).

The neurotrophins bind with high affinity to the trk family. There are three types of receptor genes in this family, Trk A, Trk B and Trk C (Huang and Reichardt, 2001). All Trk receptors display high conservation in their intracellular domains. NGF binds

specifically to Trk A receptor, BDNF and NT-4/5 bind specifically only to Trk B. NT-3 exhibits to bind all three receptors, but act with highest affinity to Trk C receptor (Lamballe et al., 1991, Barbacid, 1994, Ryden et al., 1995, Ryden and Ibanez, 1996). All Trk receptors display high conservation in their intracellular domains, including the catalytic tyrosine kinase domain and the juxta-membrane domain. The Trk receptors also demonstrate a number of truncated isoforms. It was found that there is no similarly between the Trk and p75 receptor ligand-binding or cytoplasmic domain, thus, they generally have opposite consequences (Meakin et al., 1992, Patel et al., 2000).

All of the neurotrophins bind and act with much lower affinity to p75 receptors compared to Trk receptors (Rodriguez-Tebar et al., 1990, Liepinsh et al., 1997) . The neurotrophin receptor p75 is found to be expressed in many areas of the brain, such as basal forebrain, septum, olfactory bulb and cerebellar purkinje cells (Koh et al., 1989, Yan and Johnson, 1989). Although p75 was the first isolated neurotrophin receptor, the role of its signaling pathway is not fully understood compared to Trk neurotrophin receptors (Johnson et al., 1986).

There is now much evidence that, unlike embryonic neurons, adult neurons in the CNS have limited capacity to elongate growth cones and regenerate their axons. Therefore, there has been a great interest shown in the identification of environmental factors that would facilitate the intrinsic properties of neuron. It has been postulated by (Ramón y Cajal, 1928) that the lack of neurotrophic factors inhibits axon growth in CNS. Neurotrophic factors are growth factors that contribute to growth and development of the CNS and PNS (Twiss et al., 2006). These factors are not generally produced during adulthood unless there is an injury to the CNS

(Barde, 1988, Bregman, 1998). Studies have shown that regeneration of growing axons can be promoted by the application of neurotrophins (Logan et al., 1992, Logan et al., 2006). Each neural type is sensitive to different neurotrophic factors or combination of many factors (Lu and Tuszynski, 2008) (See Table 1.1 and Appendix 9).

The first known neurotrophic factor was nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1951, Levi-Montalcini, 1987). NGF can be introduced into the intact lesion spinal cord using gene delivery techniques (Lu and Tuszynski, 2008). The axons were induced to grow by injecting NGF into the spinal cord. Fibroblasts genetically modified to secrete NGF were injected into either central grey matter of the lesion or the non- lesion spinal cord. It has been found that there was a similar pattern of axonal responsiveness to NGF in both lesion and non-lesion site. This finding indicated that axons retain their sensitivity to growth factors (Tuszynski et al., 1994).

Over subsequent years, numbers of neurotrophic factors have been identified such as neurotrophin-3 (NT-3), neuotrophin-4/5 (NT-4/5), brain-derived nerve growth factor (BDNF), glial cell derived nerve growth factor (DGNF), leukemia inhibitory factors (LIF) and ciliary neurotrophic factor (CNTF) (Barde, 1989, Lu and Tuszynski, 2008).

In a series of studies, several groups have reported that BDNF could help prevent atrophy of neurons as well as their regeneration (Kobayashi et al., 1997, Lu et al., 2001, Lu and Tuszynski, 2008). Interestingly, the study by Kobayashi (1997) explained that even though BDNF helped prevent the death of neuronal cell bodies,

it did not influence the growth of corticospinal axons in the spinal cord. The limitation of this treatment might be that BDNF and Trk B receptor was not trafficked from cortical soma to the axons (Kobayashi et al., 1997).

A slightly different effect has been observed in measuring responsiveness of axons to NT-3. In 1994, Schnell and colleagues demonstrated that a single injection of NT-3 above the injured site in a spinal cord combined with the Nogo neutralizing antibody IN-1 dramatically increased the distance of axons extension in the thoracic dorsal hemi section (Schnell et al., 1994). However, this treatment did not influence the regrowth of severed axons across the lesion site and into the distal host tissue. Significantly, NT-3 promotes the growth of upper motor neurons in corticospinal axons as well as ascending dorsal column sensory tracts. The presence of the Trk C receptor may be responsible for regrowth stimulation of NT-3 on injured corticospinal tracts (Grill et al., 1997).

It has also been reported that prolonged delivery of GDNF significantly increases the sprouting of corticospinal axons (Deumens et al., 2005). When GDNF was injected in compression injuries, the axons were stimulated and regrew into white matter. This suggested that GDNF transfer to a lesion site is sufficient for axon regrowth into the distal host spinal cord. Yet, the regenerating fibres were not blocked by myelin in the caudal sites. Even though GDNF delivery has a very potent effect in stimulating the regrowth of the axons, unfortunately, GDNF family receptor a-1 (GFRa-1) has not been shown in corticospinal neurons. The growth-stimulating effects of neurotrophins are partly regulated by increasing the intracellular cAMP and activating protein kinase A (PKA). Therefore, axonal regeneration might be increased by other manipulations that raise cAMP concentrations (Deumens et al., 2005).

Table 1.1: Table of selected literature showing the sensitivity of spinal cord axons to various neurotrophic factors.

Growth Factors	Injured axons (locations)	References
NGF	Nociceptive spinal axons	(Tuszynski et al., 1994, Tuszynski et al., 1996)
	Cerulospinal axons	(Tuszynski et al., 1994, Tuszynski et al., 1996)
BDNF	Reticulospinal axons	(Ye and Houle, 1997)
	Vestibulospinal	(Jin et al., 2002)
	Local motor axon	(Lu et al., 2001)
	Local sensory axon	(Lu et al., 2001)
NT-3	Corticospinal axons	(Schnell et al., 1994)
	Dorsal column sensory axons	(Bradbury et al., 1999)

NT-4/5	Local motor axons	(Blesch et al., 2004)
	Coerulospinal axons	(Blesch et al., 2004)
GDNF	Dorsal column sensory	(Blesch and Tuszynski, 2003)
	Local motor axons	(Blesch and Tuszynski, 2003)
	Propriospinal axons	(Blesch and Tuszynski, 2003)

This table is modified from (Lu and Tuszynski, 2008).

1.18 ANTIBODIES TO GROWTH INHIBITION MOLECULES

Oligodendrocytes and astrocytes are present in the CNS after injury as a result of an inflammatory response. Both of these cell types express growth inhibitory molecules to axons (Yang et al., 2006). Therefore, axons can only regenerate if they have no receptors that bind to these molecules or if they can bypass the signals released. It has been reported that embryonic neurons are able to grow over oligodendrocytes increasing the calcium level in their growth cones, resulting in the collapsing of the cones (Carmen et al., 2007).

Astrocytes produce proteoglycans that block the axonal regeneration by inhibiting the growth promoting features of laminin and other ECM molecules. Inhibiting factors released by oligodendrocytes and myelin can be blocked by using an antibody such as IN-1 (Caroni and Schwab, 1988). IN-1 was developed to counter the effects of two myelin-associated inhibitory proteins for neurite outgrowth (NI-250 and NI-35) These antibodies have proven to be effective in improving axonal regrowth in mature higher vertebrates (Spillmann et al., 1998).

1.19 GUIDANCE MOLECULES

During axonal development, guidance cues within the CNS play a major role in shaping the formation of neural circuits (Anderson et al 2008). With the cessation of development, guidance cues may be lost, reduced, or altered. Injury to CNS can lead to increased inhibition at a lesion site. It may be possible that recapitulation of the developmental guidance process in the injured CNS allow enhanced functional recovery (Bolsover et al., 2008, Giger et al., 2010).

1.19a) Ephrins

Ephrins (Eph) are important contact-dependent regulators of axonal guidance during development and have a repulsive effect (Pasquale, 2005). These receptors are expressed on corticospinal neurons. Ephrin B2 is regulated around lesion sites, while Ephrin B3 is expressed mostly in myelin around axons. Several studies by (Goldshmit et al., 2004, Goldshmit et al., 2006, Bolsover et al., 2008) have demonstrated that Eph signalling enhances recovery and neuronal sprouting.

The role of Eph signaling in limiting axonal regeneration was studied by (Bolsover et al., 2008). The experiment was conducted using EphA4-null mice with lateral hemisections of the cord at T12. The study of axon regeneration was obtained by injecting Fluoro-Ruby into cervical cord to anterogradely label descending axons and by injecting Fast Blue into lumbar enlargement to retrogradely label the cell bodies of neurons with regenerating axons. The result strongly indicated that signalling via EphA4 is one of the most significant contributors to the failure of axonal regeneration in spinal cord (Fabes et al., 2007). The main mediators of ephrin-induced repulsion are the Rho family of small GTPases, particularly RhoA (Kullander et al., 2001). It is activated by guanine nucleotide exchange factor, ephenix (Sahin et al., 2005). This suggests that the Ephrin signaling is complicated and a co-expression of ligands can alter the receptor sensitivity. Thus, the co-expression may have a significant role in the nervous system (Iwamasa et al., 1999).

1.19b) Semaphorins and their receptors

Semaphorins (Sema) belong to a family of proteins that share a conserved 500 amino acid motif termed the "sema" domain. There are several classes of

semaphorins based on the species in which they are expressed and whether or not they are membrane bound or secreted. Semaphorins are present in the intact myelin and at lesion sites in the CNS and their receptors are expressed by several axons in the spinal cord (Bolsover et al., 2008). A study by Pasterkamp (2001) suggested that Semaphorins can act as an attractive or repellent cue, depending on the presence of CSPG and neuropilin expression. Semaphorin receptors include plexins and rneuropilins 1-2 (Pasterkamp and Verhaagen, 2001, Bolsover et al., 2008). Unlike many types of semaphorins which act as repulsive cues, Semaphorins 7A (Sema 7a), a membrane-anchored member of the semaphorin family that could have an immuno-modulatory effect and facilitate neuronal function. The evidence shows that sema 7A promoted axonal growth in the central and peripheral sections of the brain and is essential for correct axon tract formation during embryonic development (Horner 2000). It is also thought to enhance axonal regeneration through integrin receptors and the activation of MAPK signaling pathways (Pasterkamp and Verhaagen, 2001).

Interestingly, it was found that sema 3 could act as a polarizing factor for dendrite development in cultured hippocampal neurons. The polarization of dendrites is important for neurons to function in the nervous system. This study has demonstrated that exposure of the undifferentiated neurite to localized sema 3a suppressed its differentiation into an axon and promoted dendritic network and formation.

Sema 3 is the class of semaphorins that has been most investigated because of its role in growth cone collapse (Pasterkamp and Verhaagen, 2001). It has been found that semaphorin 3's and their receptors are expressed in the mammalian spinal cord

after injury and it has suggested that this may be one of the crucial factors that may inhibit/promote axonal regeneration (Bolsover et al., 2008). Semaphorins are present in the intact myelin and at lesion sites in the CNS. The receptors are expressed by several axons in the spinal cord (Bolsover et al., 2008). It is evident that further studies are required to determine the importance of semaphorins in terms of limiting and promoting axonal regeneration in the spinal cord.

1.19c) Netrins and Slits in the mammalian spinal cord

Netrins and Slits are potent axon guidance molecules during development which act as repulsive molecules in the intact CNS and at the site of injury (Barallobre et al., 2005, Bolsover et al., 2008). Netrins are composed of a small family of secreted laminin related molecules acting as chemoattractants or chemorepellents depending on the neurons. To date, four members of the netrin family have been identified in rodent which are Netrin-1,-3,-4 (ß-Netrin), and G-Netrin (Barallobre et al., 2005). Netrin-1 acts as a survival factor and has the ability to bind to members of the Deleted in Colorectal Cancer (DCC) and UNC5 families (Netrin receptor) (Bolsover et al., 2008). Studies by (Brose et al., 1999, Bolsover et al., 2008) demonstrated that netrin-1 induced interaction of UNC5B with the brain specific GTPase PIKE-L. This interaction is thought to trigger the activation of PtdIns-3-OH kinase, preventing UNC5B's pro-apoptotic activity and promotes neuronal survival. However, this is in contrast with other studies as Netrin, Slit and their receptors are known to inhibit axon outgrowth or be involved in the formation of CNS scar (Bolsover et al 2008, Curinga et al 2008, Brose et al 1999).

Table 1.2: Summary of molecules identified as Inhibitory to CNS axonal regeneration.

Factors	Cell type	Location	Author/Group
MAG	Oligodendrocytes	CNS myelin PNS myelin	(Mukhopadhyay et al., 1994)
Netrin-1/2	Oligodendrocytes	Ventral spinal cord	(Serafini et al., 1994)
Semaphorins	Muscle cells Neurons	Neurons	(Luo et al., 1993)
N1-35/250	Oligodendrocytes	CNS myelin	(Schwab et al., 1993)
CSPG	Astrocytes	Scar tissue	(Snow et al., 1991)

EMBROYNIC

Axonal Growth: Axonal guidance, long distance extension

Regulators: semaphorins, slits, ephrins, netrins

Receptors: neurophilins, robos, ephs, DCC/UNC-5

Axonal Growth: refinement of connectivity

Regulators: neurotrohins, glutamate

Receptors: Trks

Axonal Growth: limited plasticity, short sprouting

Regulators: Nogo MAG, OMgp, CSPG ephrins

Receptors: NgR, CSPG receptors, ephs

ADULT

Figure 1.5: A schematic summarising the shift of CNS axons through three phases of development.

The table shows the extrinsic control of axonal regeneration from developmental to adult stage. As axons become mature, the plasticity is limited and many inhibitory molecules such as Nogo, MAG, OMgp and CSPGs play crucial roles in limiting regeneration. *Adapted from* (Liu et al., 2006)

1.20 FACTORS THAT LIMIT REGENERATION IN CNS

1.20a) Extracellular factor (CNS inhibitors)

Myelin is produced by oligodendrocytes in the CNS; it is a layered tissue that sheathes the axons (Vinson et al., 2001). This myelin sheath wraps around axons, allowing the rapid conduction of action potentials to occur. Mature oligodendrocytes form complex morphologies with extended membrane structures that ensheath multiple axons (Quarles, 2005). Traumatic injuries to both brain and spinal cord result in substantial axonal damage. During this trauma, the neuronal cell body and the axon proximal segment of an injured site often survive, while the axon distal segment undergoes degeneration process. The axonal loss interrupts the biochemical balance between axons and oligodendrocytes. In addition, it can indirectly lead to the death of oligodendrocytes and accumulation of cell debris. CNS myelin contains various inhibitors of growth which contribute to the regenerative failure of severed axons. With an increasing number of CNS inhibitors identified, an important question is to define their mechanisms of action and the ways to block the growth inhibitor (Bahr and Bonhoeffer, 1994, Schwab et al., 2006).

1.20b) Myelin-associated Glycoprotein (MAG)

The myelin associated glycoprotein (MAG) contains a sialic acid binding site with five extracellular IgG like domains, identifying MAG as one of subgroup of the immunoglobulin family. It is selectively found in periaxonal Schwann cell and oligodendroglial membranes of myelin sheaths which serves a function in glial-axon interactions in the PNS and CNS. MAG is a bi-functional molecule that facilitates cell process outgrowth. Myelin proteins are expressed abundantly during the

developmental period when axons are in an active period of growth and extension. However, MAG is also thought to strongly inhibit axonal and dendritic outgrowth in adult life as well as neurite growth in tissue culture.

In PNS, MAG contributes to the limitation of myelination by Schwann cells and maintains the interaction between the axon and its myelin sheath (Quarles, 2009). This interaction is essential to sustain normal axonal morphology. Myelination of peripheral axons by Schwann cells usually occur in the absence of MAG. The disruption of axon-myelin interaction, however leads to loss of myelin compaction (Ide, 1996).

A recent study by Hannila (2008) gave further insight into the MAG pathway by indicating that regulated intramembrane proteolysis of p75 NTR is required for MAG-mediated activation of Rho (Hannila and Filbin, 2008). The neurons were treated with soluble MAG which induced the cleavage of the p75 NTR extracellular domain by α-secretase. This was followed by a PKC-dependent α-secretase cleavage within the transmembrane domain into MAG pathway indicating the regulation of intramembrane proteolysis which releases the intracellular domain into the cytoplasm. It is suggested that cleavage of p75 NTR is crucial for both activation of Rho and inhibition of α. γ-secretase blocks these events leading to increased neurite outgrowth. On the other hand, expression of the cytoplasmic domain of p75 NTR was adequate to induce the activation of Rho and inhibit neurite outgrowth on a permissive substrate (Quarles, 2009).

1.20c) Nogo

Nogo has been identified as a component of the CNS myelin that restricts regeneration of the axon in adult vertebrates. Nogo-A is a membrane protein with 66 amino loop structure located between two transmembrane domains. This is known as Nogo-66 (Chen et al., 2002, Domeniconi et al., 2002). It is expressed in the adult CNS mammalian on the surface of oligodendrocytes and functions by binding to an axonal Nogo-66 receptor (NgR). The splice form, Nogo-B is found in many tissue and cell types including adult neurons, whereas Nogo-C is expressed highly in muscle. The neural expression of Nogo-A is prominent during the development of the adult nervous system and found on the surface of many cells. Nogo-A has been shown to be a potent inhibitor of axonal growth and induces growth cone collapse. The functions of Nogo-B and Nogo-C, however, are currently unknown. All three main domains of the gene encoding have different length but share a sequence of 188 amino acids at their Carboxy (C)-terminus. NgR is a GPI-linked LRR protein expressed in multiple types of neurons.

Nogo-A has been shown to induce neurite growth inhibition, growth cone collapse, and inhibition of fibroblast (Schwab et al., 2006). Currently, only one binding site of Nogo-A has been identified which is the 443-residue glycosyl-phosphatidylinositol-linked, leucine-rich repeat glycoprotein NgR (This part of the receptor binds to the region of 66 amino acid at the C-terminus) (Domeniconi et al., 2002, Hu et al., 2005). Interestingly, NgR also binds to neurite growth inhibitory myelin proteins MAG (Fournier et al., 2002).

The strong inhibitory role of CNS myelin can be partially neutralised by antibodies such as Nogo-A, Nogo gene deletions, soluble NgR fragments and NgR blocking peptides. Neutralising Nogo-A antibodies have been shown to significantly reduce the blocking activity of myelin. Monoclonal antibody (mAb) IN-1 is the most frequent antibody used against Nogo-A (Caroni and Schwab, 1988). A study by Schwab (2004) demonstrated that injection of IN-1 Fab fragment against Nogo-A specific active site into the intact adult rat cerebellum induces sprouting axons and expression of growth-related genes in Purkinje cells. This suggests that neutralising Nogo antibodies can induce a growth response in the intact adult CNS (Schwab 2004).

1.21 A ROLE OF cAMP IN REGENERATION OF THE ADULT MAMMALIAN CNS

Recent studies have suggested that there is a direct correlation between cellular cyclic adenosine monophosphate (cAMP) levels and the inhibition of neurite outgrowth. Elevation of cellular cAMP in the neurons following injury can induce axonal regeneration in the CNS (Spencer and Filbin, 2004). cAMP is not only known to increase the growth capacity of injured axons, but also partly responsible for overcoming CNS myelin inhibition (Hannila and Filbin, 2008). The experiment investigated by (Qiu et al., 2002) demonstrated that 24 hrs after sciatic nerve lesion, cAMP levels in DRG neurons increased by two-fold. This effect was inhibited by treatment of PKA inhibitors, which indicated that the lesion effect is initially dependent on PKA activity. A week after the lesion, the effect became PKA-independent and cAMP levels returned to baseline level. The findings suggest that

cAMP mediates its effect through a series of signaling of PKA dependent and independent pathways. Upon becoming PKA-dependent, the effects of cAMP became transcription dependent and required the transcription factor cAMP response element binding protein (CREB). CREB is activated by elevated levels of cAMP and acts as a primary mediator of cAMP-induced transcription (Spencer and Filbin, 2004). Interestingly, CREB activity is necessary for overcoming inhibition by MAG and myelin. The activation of CREB is sufficient to promote axonal regeneration (Gao et al., 2004). This has led to the assumption that the activation of CREB by cAMP causes the transcription genes to participate in overcoming myelin inhibition. The cAMP- regulated genes and enzyme such as Arginase 1, neuropeptide Y, cAMP-response element modulator (CREM), VGF nerve growth factor-inducible growth factor (VGF) and IL-6 are known to play a role in overcoming myelin inhibition. It was thought that Arginase-1 enzyme may involve in this pathway and may serve as one of the most emerging key for studying myelin inhibition effect (Costigan et al., 2002).

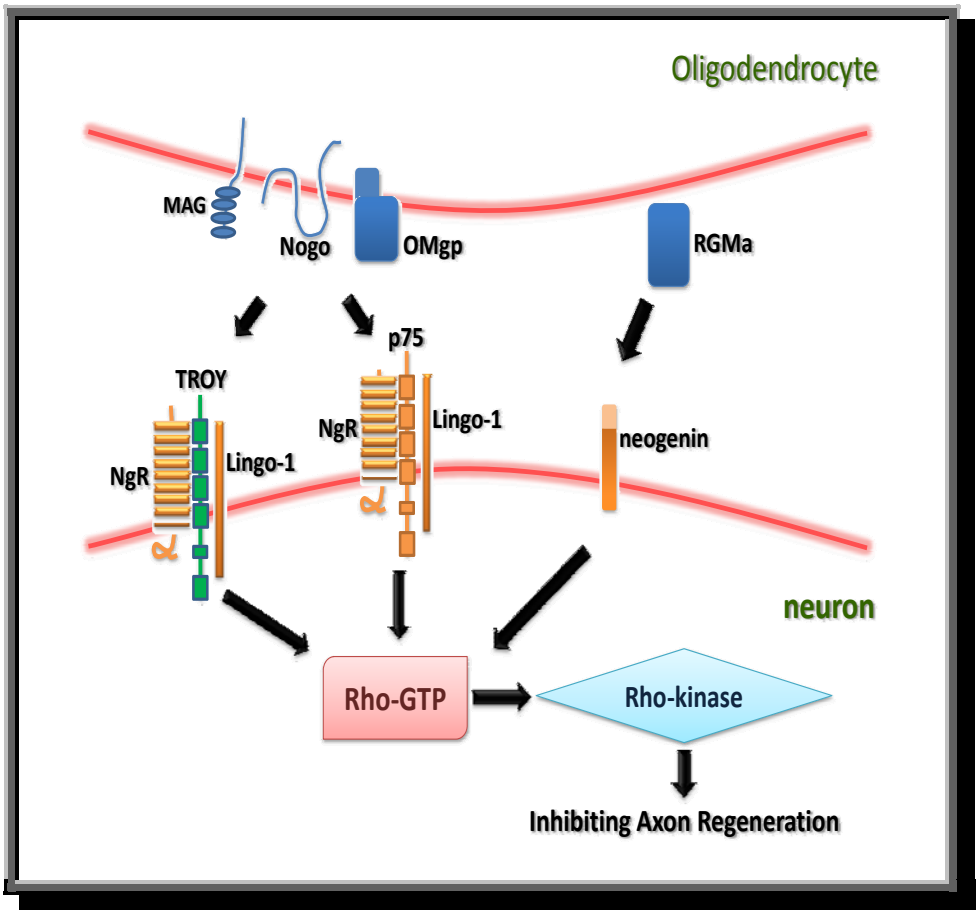

Oligodendrocyte
MAG
Nogo
OMgp
RGMa
p75
TROY
NgR
Lingo-1
NgR
Lingo-1
neogenin
neuron
Rho-GTP
Rho-kinase
Inhibiting Axon Regeneration

Figure 1.6: Signaling Mechanism of Axon Growth Inhibition.

Nogo-A, MAG and OMgp are the main inhibitors of neurite growth in CNS myelin, interacting with a receptor complex composed of NgR; p75 to stimulate Rho activity. Rho-Kinase, a downstream effector of Rho-A, are key signals that elicit axon inhibition. *Adapted from* (Hata et al., 2006)

1.22 ADULT ZEBRAFISH AS A MODEL FOR CNS REGENERATION

Adult teleosts fish, in contrast to mammals, are capable of regenerating axonal tracts as well as cells and entire tissues in the CNS, leading to recovery of function (Becker and Becker, 2008). In many studies, zebrafish (*Danio rerio*) has become established as an experimental model to study regenerative axonal growth of neurons projecting to the spinal cord. One of the main reasons that fish are able to regenerate axons is thought to be due to the conducive environment of their CNS (Becker et al., 1997). The CNS of teleost fish appears to contain few inhibitory molecules and a number of promoting molecule for axon growth. For instance, Nogo-A has a role in inhibiting neurite growth in mammals and it does not play any role in axonal regeneration of teleost fish (Diekmann et al., 2005). This study demonstrated that after crushing of the optic nerve, retinal ganglion cells (RGCs) are able to regrow, as indicated by the re-expression of a number of genes that had previously been down regulated during development (Becker and Becker, 2007). One of the genes expressed is the growth cone-associated protein-43 (GAP-43). GAP-43 is known to be correlated with CNS axonal regeneration. It is concentrated in the cortical cytoskeleton of axonal growth cones and interacts with the cell membrane and actin filaments. Over-expression of the genes results in enhancement of neurite outgrowth *in vitro* (Udvadia et al., 2001).

The CNS environment of zebrafish is not just growth permissive because of the lack of inhibitory molecules, but might also actively promote axon regrowth (Udvadia et al., 2001). It was found that zebrafish oligodendrocytes can express an array of growth-promoting cell surface molecules of the immunoglobulin super family, as well as a cell adhesion L-1 homolog. In mammals, Schwann cells express L-1,

suggesting that fish CNS may share similar features with Schwann cells of PNS in terms of their capacity to support axonal regeneration (Thallmair et al., 1998).

In summary, the mammalian CNS exhibits very limited capacity for spontaneous regrowth of axon tracts after injury. Recent evidence suggests that the failure of successful axonal regeneration is not due to the inherent property of neurons, but mainly due to the environment encountered by re-extending axons (Fawcett, 2006). This inability for regeneration in CNS stands is in sharp contrast to the situation seen in both fish and mammalian PNS. Recent studies in this area have focused on varying the post-lesion microenvironment within the CNS. In an attempt to promote axon growth, these studies have included the use of neurotrophic factors, the blocking of myelin-associated inhibitory molecules as well as the development of guidance molecules (Jones et al., 2003, Becker and Becker, 2007). Important advances are being made in this research area, however, there are still many mechanisms involved in the process of axonal growth to be elucidated. The failure of axons to regenerate after injury remains one of the greatest challenges in the field of brain and spinal cord injury repair.

1.23 HYPOTHESIS

1) It is hypothesised that neurons in the adult mammalian CNS fail to regenerate after injury because of the non-permissive environment surrounding the injured axons.

2) It is hypothesised that axonal regeneration in zebrafish is possible because of the absence of such inhibitory molecules.

3) It is hypothesised that growth of neurites from zebrafish CNS neurons can be influenced by the presence or absence of certain neurotrophic factors.

4) It is hypothesised that mammalian neurons should be able to regenerate their axons if they could be grown in an environment provided by the zebrafish CNS.

1.24 AIMS

This study aims to identify the factors and mechanisms involved in axonal regeneration in lower vertebrate species (Adult Zebrafish *Danio rerio*). It is hoped that a deeper understanding of why zebrafish CNS neurons are capable of axonal regeneration whereas mammalian CNS neurons are not, will allow us to design better strategies in promoting the regeneration of adult mammalian CNS neurons. The specific aims were.

1) To develop methods to isolate and grow adult zebrafish CNS neurons in tissue culture.

2) To examine whether or not neurotrophic factors play a role in promoting neurite growth of adult zebrafish neurons.

3) To examine the effects of different substrates on adult zebrafish neurite growth.

4) To investigate the growth of zebrafish CNS neurons on various tissue substrates from rat sections using a cryoculture technique.

CHAPTER TWO

GENERAL METHODS

2.1 ANIMALS

2.1a) Adult zebrafish

Adult Zebrafish (*Danio rerio*) of approximately 3-4 cm in body length and aged from 8 to 12 months old (determined by body length and morphological appearance) were obtained from several local pet shop aquariums. After purchase, the animals were kept in colonies of 15 in each tank at the Bond University laboratory. The maintenance procedures used to house the Zebrafish were strictly followed from a standard protocol (Westerfield, 2000). Briefly, all tanks were well-aerated with tap water at 28°C with a 14/10 hour light and dark cycle. The water was maintained by recirculating system and routinely filtered chemically and biologically and pH was adjusted to 6.5-7.5. The water was passed through charcoal and paper filters and irradiated with ultraviolet light (UV) in a continuous cycle. Zebrafish were fed daily at the beginning of the light cycle with commercial tropical aquarium fish food. The total number of adult zebrafish used in each study is given in Appendix 1.

Prior to each experiment individual Adult zebrafish were anesthetised for 5 minutes by adding 4mg/ml ethyl-3-aminobenzoate methane sulfonate (Tricaine, Sigma St. Louis, MO) to the water in a holding tank when required. Once anaesthetized, zebrafish were rapidly killed by decapitation and their brains rapidly dissected out.

2.1b) Rats

Adult Wistar Rats (8-12 weeks) were purchased from University of Queensland's Central Animal Breeding House. The total numbers of rats used throughout all experiments are shown in Appendix 1.

2.2 SURGICAL PROCEDURE OF ADULT ZEBRAFISH BRAIN

The dissection method was performed under sterile conditions in a laminar flow hood. Dissection instruments were all soaked in 70% ethanol and flamed aseptically prior to dissection of the brain region of adult zebrafish. All dissections were carried out with the aid of a dissection microscope (Olympus model). Small scissors and fine forceps were used to cut the head from the rest of the body. The remaining muscle and connective tissue around the head was gently trimmed away, to expose the brain. The brain was then carefully dissected out of the skull aseptically using fine scissors and forceps, and placed in a few drops of BSF2 medium which had been previously equilibrated to 28.5°C with 1.5 % CO_2.

2.3 COATING OF COVERSLIP PREPRARATION IN TISSUE CULTURE STUDIES

Neurons were grown either on glass or plastic coverslips (13mm diameter, SMEIC; NUNC Roskilde, DK). The coverslips were dry heat sterilised in a glass Petri dish for 20 minutes in an autoclave and transferred and kept in sterile plastic Petri dishes. All coverslips were coated with poly-L-lysine (PLL) substrate. Filter-sterilised PLL (Sigma St. Louis, MO; mol wt 70,000-150,000; 20µg/ml final concentration) was applied to individual coverslips using a transfer pipette and incubated for at least an

hour at 37^{o}C. Excess solution was removed, allowing the surface to air dry. These were stored under sterile conditions until required.

2.4 PREPRATION OF MODIFIED BOTTENSTEIN AND SATO'S MEDIUM WITH 2% FOETAL BOVINE SERUM

Modified Bottenstein and Sato's medium with 2% foetal bovine serum (BSF2) is a basic culture medium for most neurons (Bottenstein and Sato, 1979). The aliquots of the individual ingredients for the preparation of BSF2 were stored at -20°C. However, Ham F-12 alone was stored at 2-8°C. An aliquot of each component was thawed at room temperature, spun down in a centrifuge, and then combined in a sterile beaker. It was ensured that a new aliquot of insulin (100 µL) was the last ingredient added to the mixture. The pH was adjusted to 7.1 using a few drops of 1M of hydrochloric acid (HCl). Once mixed, the medium was filter-sterilised using 0.22Um filter unit (Millipore Bedford, MA) into sterile 15 ml conical tubes. A preparation of BSF2 was stored at 2-4ºC for a maximum period of three weeks until required. (See Appendix 2 for the components of the medium)

2.5 A METHOD TO QUANTIFY THE EXTENT OF DENDRITIC ARBORISATION USING TEST GRID ANALYSIS

The extent of neurite regeneration on any given coverslip used for growing zebrafish neurons was quantified using a simple stereological procedure which involved the counting of intersections between growing neurites and a test grid on standardised images and micrographs. For this procedure each coverslip was in turn viewed either under a phase contrast or fluorescence microscope as appropriate. Randomly

selected, non-overlapping digital images of several fields of view were taken from each coverslip using an x40 objective and a digital camera. These images were printed onto A4 size paper at a standard magnification. The final magnification of these prints was calculated using images of a stage graticule. Each print from a given coverslip was, in turn, overlaid by a transparent film bearing an image of a test grid having vertical and horizontal test lines spaced 1cm apart. The number of intersections between growing neurites and the test grid were counted. The total number of intersections per unit length of test line applied to all the micrographs from any given coverslip was calculated. This value was taken to represent a measure of the extent of neurite arborisation for the neurons on the given coverslip (Figure 2.1).

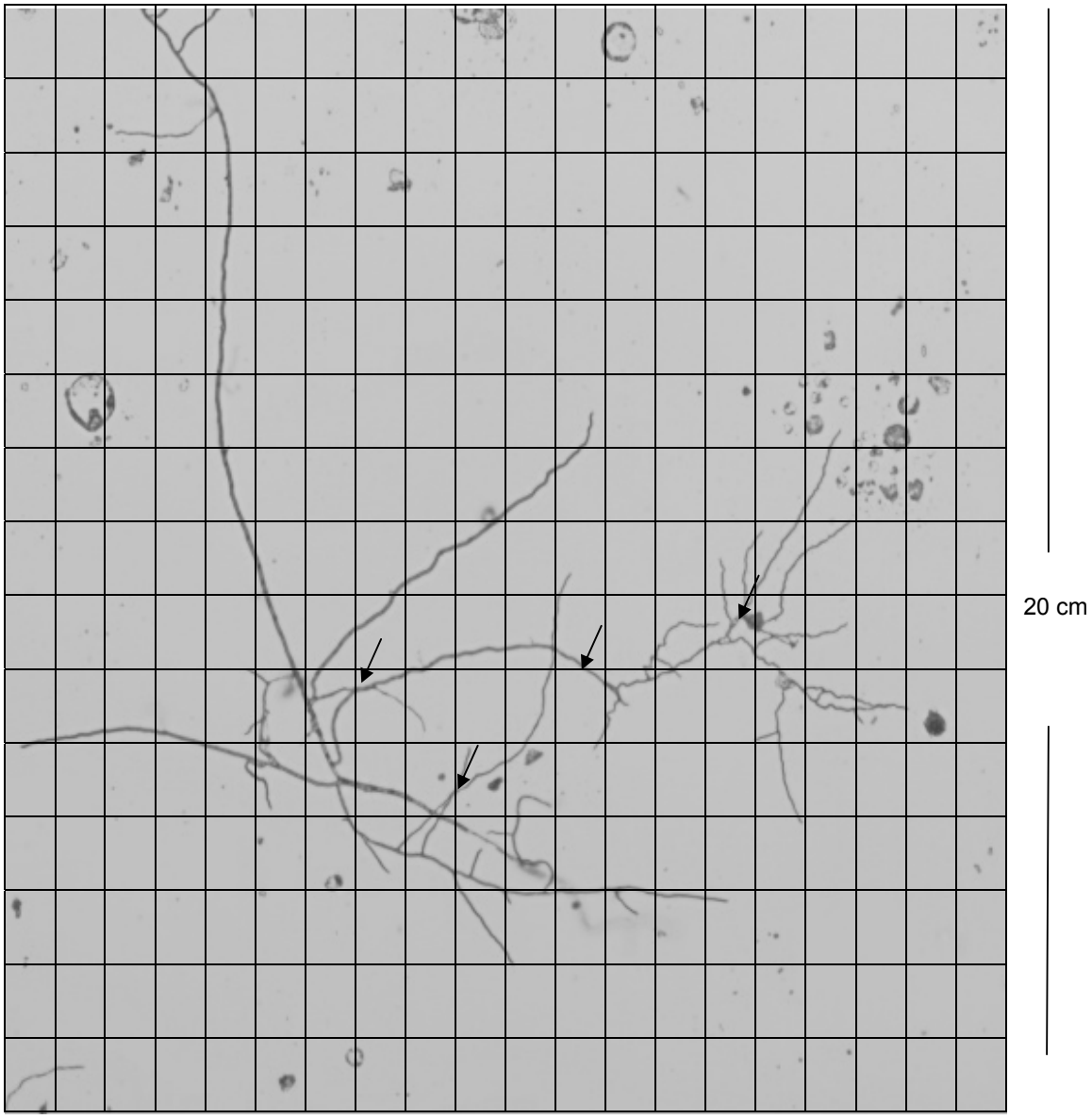

20 cm
20 cm

Figure 2.1: An example of Test grid analysis on the growing neurite regeneration.

Test grid analysis is used to quantify the morphological characteristics of adult zebrafish neurons. An image of the growing neurons was captured and placed underneath a 1cm test grid transparency sheet (20x20cm). The number of intersections between the neurites and the grid were counted both horizontally and vertically in unit test length. (See the arrows)

2.6 PREPARATION OF RAT TISSUES FOR CRYOSECTION

Tissues (brain, spinal cord, optic nerve, and sciatic nerve) were obtained from freshly killed rats. The fresh brains were separated by careful dissection into regions such as the brain stem, forebrain (here defined as the brain minus the brainstem) and cerebellum. These tissues were placed in a suitable orientation onto cork blocks before being immersed into 2-methylbutane (Sigma St. Louis, MO), and cooled in liquid nitrogen for few minutes. This led to the rapid freezing of the tissues. These frozen blocks of tissues were stored for up to six months in the freezer at -80ºC, until required for sectioning.

2.7 PREPARATION OF CRYOSTAT SECTIONS OF BRAIN TISSUES

The blocks of frozen tissues which had been prepared as described above were attached to the chuck of a cryostat (American Optical Corporation) using OCT compound (Tissue-Tek Sakura Prohosp, DK). Frozen sections were cut at a thickness of 10μm in the cryostat chamber which was maintained at a temperature of approximately -18ºC. Each section was picked up on to a sterile coverslip previously coated with Poly-L-lysine and this was placed individually in a chamber of a sterile 24-well culture plate holder (Costar Van Nuys, CA). When all the chambers in a given 24-well plate were full, they were stored at -80°C until required.

2.8 IMMUNOHISTOCHEMISTRY STAINING OF NEURONAL CELL CULTURES

Neuronal cell cultures were allowed to grow for a period of seven days before they were fixed and stained. The cells were fixed in 2% paraformaldehyde (PFA) for 20 minutes at 4°C. Prior to immunohistochemical staining, the fixed cells were washed in three separate changes 1µl of PBS and then post-fixed in cold (-20°C) 100% methanol for up to 10 minutes. The antibodies were diluted with BSA prior the staining. Primary antibody was normally applied to the section for 3 hours at room temperature. The antibody was then washed off in three changes of PBS. Subsequently, a secondary conjugate fluorescence antibody (Sapphire Bioscience, NSW, AUS) were added for one hour and washed off with PBS. At the completion of the immunolabeling procedure, the coverslip was then inverted onto a drop of anti-fade mounting on a glass microscope slide and sealed with nail varnish. Photographs of the immmunolabeled culture were taken using a SPOT digital camera connected to Olympus BH-2 microscope. A full protocol is provided in Appendix 5-6.

2.9 STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS for Window version 17.0.0 (SPSS Inc, Chicago, III). Analysis of variance (ANOVA) procedures were used in most analysis unless otherwise indicated. A probability value of less than 0.05 ($p<0.05$) was used to determine the significance or otherwise of any given analysis.

CHAPTER THREE

DETERMINATION OF THE OPTIMAL CONDITIONS FOR THE CULTURE OF ADULT ZEBRAFISH CNS NEURONS

3.1 INTRODUCTION

The central nervous system (CNS) neurons of lower vertebrate such as teleost fish are capable of regenerating new neurons after injuries. These species have a distinctive ability to replace damaged neurons using newly generated neurons (Lie et al., 2004). Higher vertebrate species such as mammals are generally incapable of such spontaneous regeneration of neurons after lesions (Becker et al., 1998b, McBride et al., 2009, Buckley et al., 2010) in adult life. The olfactory bulbs and the hippocampal formation appear to be the only regions capable of generating new neurons during adult life (Perlmutter and Anderson, 1994). However, the recent discovery of neural stem cells in the adult mammalian brain opens up the possibility that they could be stimulated to be able to produce new neuronal cells to replace those lost as a result of degeneration or injury (Connaughton and Dowling, 1998, Huang et al., 2009).

In contrast to the mammalian brain, many proliferation zones have been identified in the adult brain of teleost fish. The two particular areas of proliferation that have been of interest to neurobiologists are the olfactory bulb and lateral posterior zone of the dorsal telencephalon (Goldman-Rakic, 1980, Doherty et al., 1987). It is thought that part of the dorsolateral telencephalon of teleosts is homologous to the mammalian hippocampus. The vast majority of new neurons in teleost fish species are not only generated in structures homologous to the hippocampus and olfactory bulbs, but also in dozens of other brain areas such as telencephalon area. In some regions of the fish brain, such as the optic tectum, the new cells remain near the proliferation zones in the course of their further development (Connolly et al., 1987, Shimizu et

al., 2003), while in others, most subdivisions of the cerebellum, they migrate, often guided by radial glial, to specific target areas (Connolly et al., 1987, Cormack et al., 2008). Remarkably, with the vast mitotic division activity and well- defined proliferation zones in adult teleost brain, it has been shown that adult zebrafish are able to generate cells in proliferation zones at least 1-2 magnitude higher than in the mature mammalian brain (Otto et al., 1987, Lois and Alvarez-Buylla, 1994). Approximately 50% of the young cerebellar cells undergo apoptosis, whereas the others survive for the rest of the fish's life (Zupanc 2006). A large number of the surviving cells differentiate into neurons (Raivich and Kreutzberg, 1987, Siigur et al., 1987, Whittemore et al., 1987, Zupanc, 2006a).

A further difference between zebrafish and mammals is their ability to continuously regenerate neuronal axons within the CNS after being severed or lost due to cell death. Moreover, newly generated axons of zebrafish are capable of finding their appropriate targets and forming synaptic contacts to restore function (Whittemore et al., 1987, Kizil et al., 2012). These abilities have established zebrafish as a very useful model for studying axonal and neural regeneration.

Zebrafish were originally used as a model to examine the regeneration of the fin, but in recent times they have become widely used for studying the regeneration of retina, optic nerve, as well as other central nervous tissues (Lien et al., 2006, Schebesta et al., 2006, Benowitz and Yin, 2008, McCurley and Callard, 2010). In contrast to other lower species such as newts which possess similar capabilities, zebrafish are very easy to maintain and cost effective to breed in high numbers.

Given these factors, it is somewhat surprising that adult zebrafish CNS neurons have not yet been investigated in detail. In particular, there is limited research on the isolation and growth of adult zebrafish neurons in tissue culture. One aim of the present study was to examine the growth and axonal regeneration properties of adult zebrafish CNS neurons in tissue culture where other confounding factors are removed from the system. By studying neuronal regeneration of adult zebrafish, we hope to provide insight into the mechanisms that regulate and restore function after the injury and ways in which we will be able to unfold the regenerative property of neurons in adult mammalian CNS. The first part of my study focuses on developing suitable methods for isolating and growing neurons from the adult zebrafish brain in tissue culture. Some of the procedures employed and variables examined are described in this chapter.

3.2 METHODS

GROWING ADULT ZEBRAFISH NEURONS USING A TISSUE CULTURE SYSTEM

Several pilot experiments using different formulations and combinations of culture medium, temperature and enzymatic digestion were systematically examined. The media examined included BSF2 (Bottenstein and Sato 1979) and Leibovitz L-15 medium (L-15) (GIBCO Grand Island, NY). L-15 media have been used in many adult zebrafish experiments and were thought to be one of the most suitable for lower vertebrate species. The incubation temperature was varied between 28.5^{o}C and 37.^{o}C in different experiments. The tissue collected was, in some cases, treated with an enzymatic digestion procedure using collagenase (Worthington type IV, NJ) for up to 30 minutes prior to dissociation. The amount of CO_2 in the atmosphere within the incubator was varied ranging between 1.5% to 5.0% in different experiments. In these experiments, the adult zebrafish brain was dissected from freshly killed fish and collected in medium. The brain was then cut into small pieces (1mm) with a sharp sterile scalpel blade prior to incubation in the presence or absence of enzyme. The tissue was then rinsed thoroughly in fresh medium supplemented with 2% foetal calf serum (GIBCO Grand Island, NY) before being dissociated by titration in 1 ml of fresh medium. This was accomplished by passaging the tissue several times through the end of a flame-polished sterile Pasteur pipette. The dissociated cells were plated onto poly-L-lysine coated coverslips placed individually in a chamber of a 4- well-plate prior to incubation. Neurons were allowed to incubate for a period of between seven to fourteen days before examination under a phase contrast microscope to determine whether there had been any neurite regeneration.

A

B

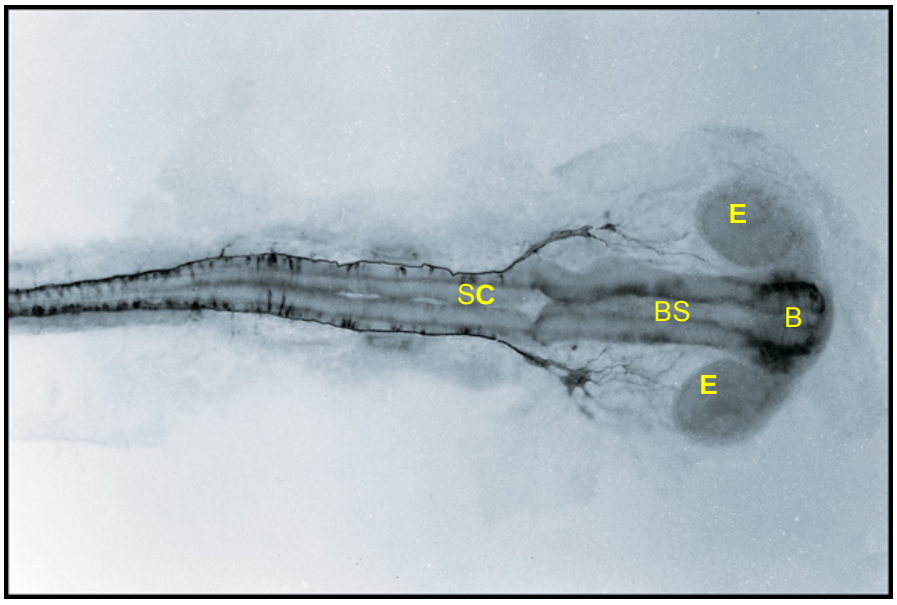


Figure 3.1: Micrographs of adult zebrafish and their brain structure.

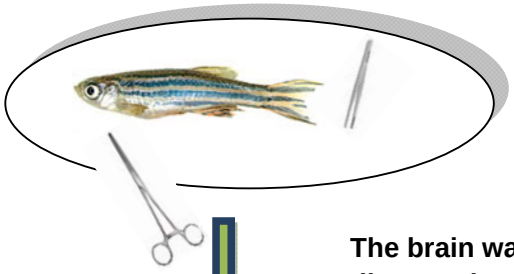
(a) Adult Zebrafish (*Danio rerio),* ranging from 8 to 12 months old were all obtained and maintained in the tank water at 28ºC for all the experiments. Scale bar = 50µm.

(b) Dissection of the adult zebrafish brain exposing, brain stem (BS), brain (B) and (SC) spinal cord. Scale bar = 50µm.

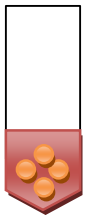
3.3 RESULTS

CULTURING OF ADULT ZEBRAFISH NEURONS

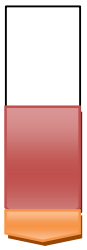
Using a phase contrast microscope, it was observed that the cells displayed neurite growth in both media and with or without enzymatic treatment with collagenase (figure 3.3-3.7). It was found that optimal growth of axons from adult zebrafish neurons occurred at an incubation temperature of 28.5^{o}C in an atmosphere containing 1.5% CO_2. Under these conditions, several clusters of neurons were seen extending their neurites from the cell bodies after 7 days in culture. The neurite growth appeared robust and showed extensive branching. It was observed that the neurons that were grown with BSF2 medium appeared to grow faster and more extensively than those cultured in L-15 medium (figures 3.3-3.6). Neurons grown in L-15 medium showed small neurite branches and appeared to have a much slower growth rate (figure 3.5-3.6). Whilst dissociated adult zebrafish neurons survived at higher temperatures and higher levels of CO_2, they generally failed to extend any neurites under such conditions.



The brain was dissected out

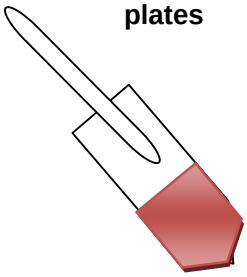


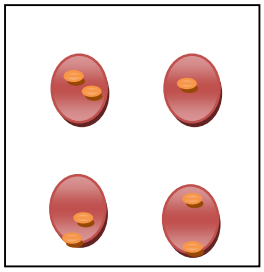
Incubated with Hanks'Solution for 20 minutes



Cells triturated with flame polished Pasteur pipette

Cell suspension plated into 4-well plates





Culture incubated at 28.5˚C and 1.5% CO_2

Figure 3.2: Growing Adult zebrafish neurons.

This diagram summarises the optimal method to dissociate and grow adult zebrafish CNS neurons in tissue culture. The whole brain was dissected out gently, prior to running incubations with other solutions. The cells were triturated several times with a sterile pipette and plated into 4-well plates at at 28.5˚C and 1.5% CO_2.

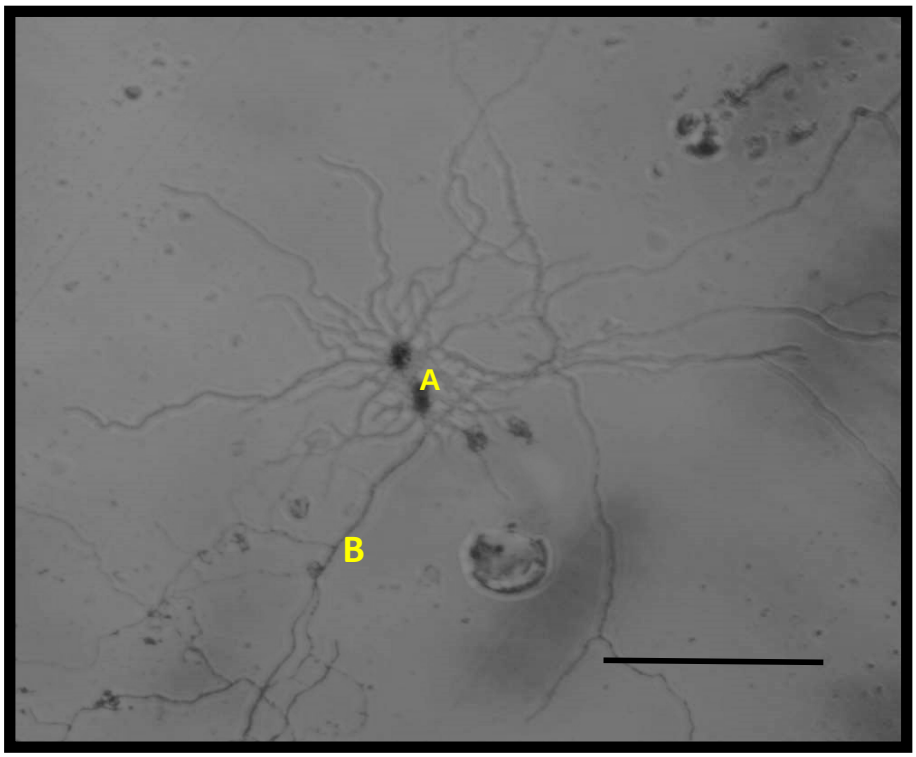

A
B

Figure 3.3 Growth of dissocated adult zebrafish neurons (7 days) in a BSF2 media at 28.5º C.

This figure shows a phase contrast of adult zebrafish neurons growing on a plastic coverslip after seven days of incubation. Several neuronal cell bodies were observed to have generated extensive neurites. (arrows) A= cell bodies ; B= neurites (scale bar = 50µm).

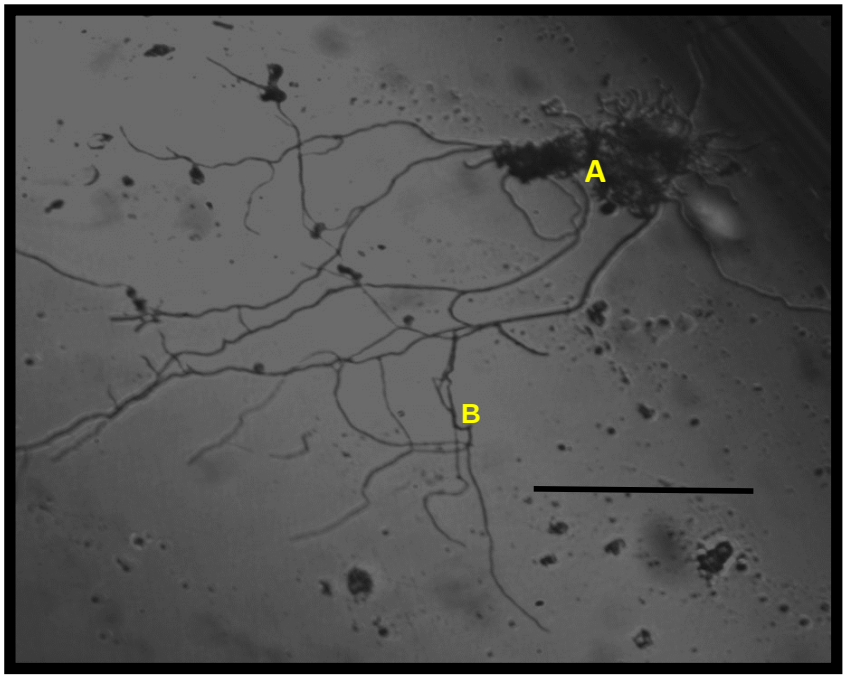

A
B

Figure 3.4: Growth of dissocated adult zebrafish neurons (14 days) with BSF2 media at 28.5º C.

Phase contrast picture of adult zebrafish neurons extending their neurites in BSF2 medium without any additional neurotrophic factors. The neurite growth appeared robust and was extensively branched in all directions. The cells were kept in the incubator for fourteen days in culture. A= cell bodies ; B= neurites (scale bar = 50µm).

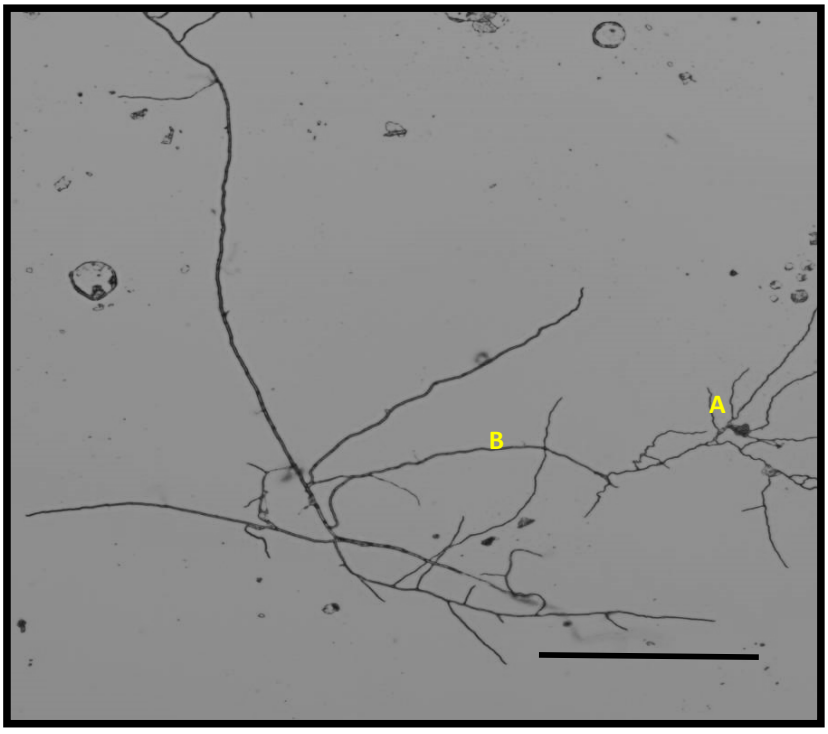

A
B

Figure 3.5: Growth of dissocated adult zebrafish neurons (7 days) with L-15 media at 28.5º C.

Phase contrast image of adult zebrafish neurons extending neurites in L-15 medium without any additional neurotrophic factors after seven days growing in culture. The neurites were extended in a short branches and some highly elongated processes from the cell bodies. A= cell bodies ; B= neurites (scale bar = 50µm)**.**

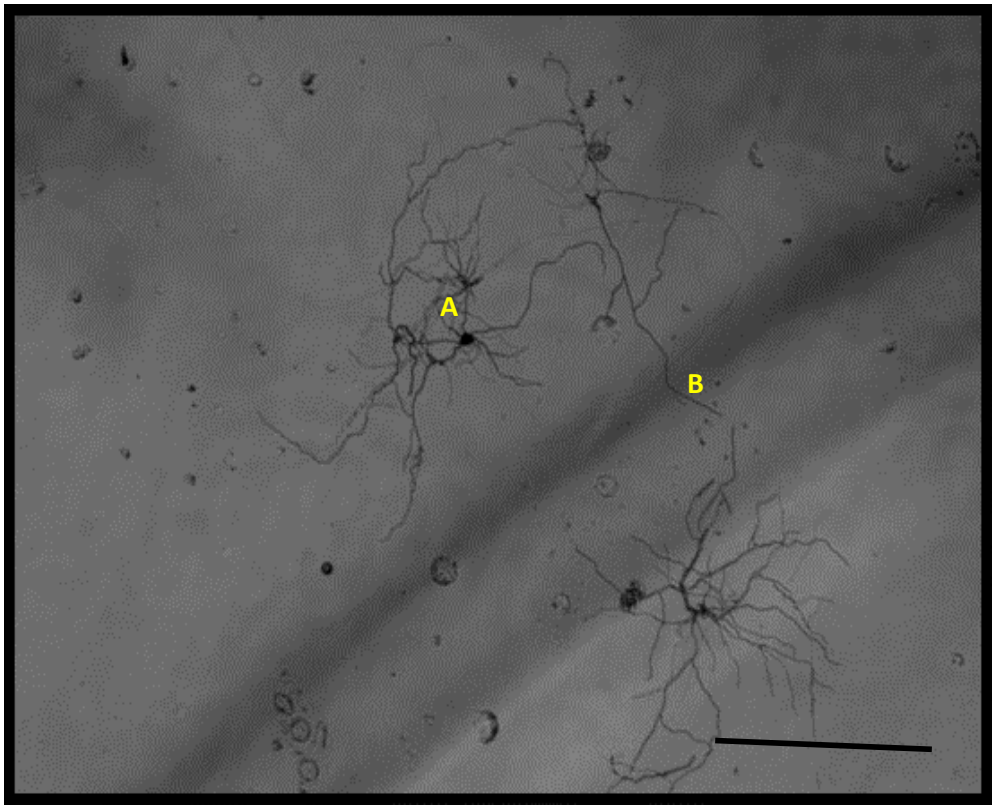

A
B

Figure 3.6: Growth of dissocated adult zebrafish neurons (14 days) with L-15 media at 28.5º C.

Dissociated adult zebrafish neurons show neurites extension on the coverslip after seven days of incubation. Several neuronal cell bodies were observed and the neurites extended their branches in a small network. (arrows) A= cell bodies ; B= neurites. A= cell bodies ; B= neurites (scale bar = 50µm).

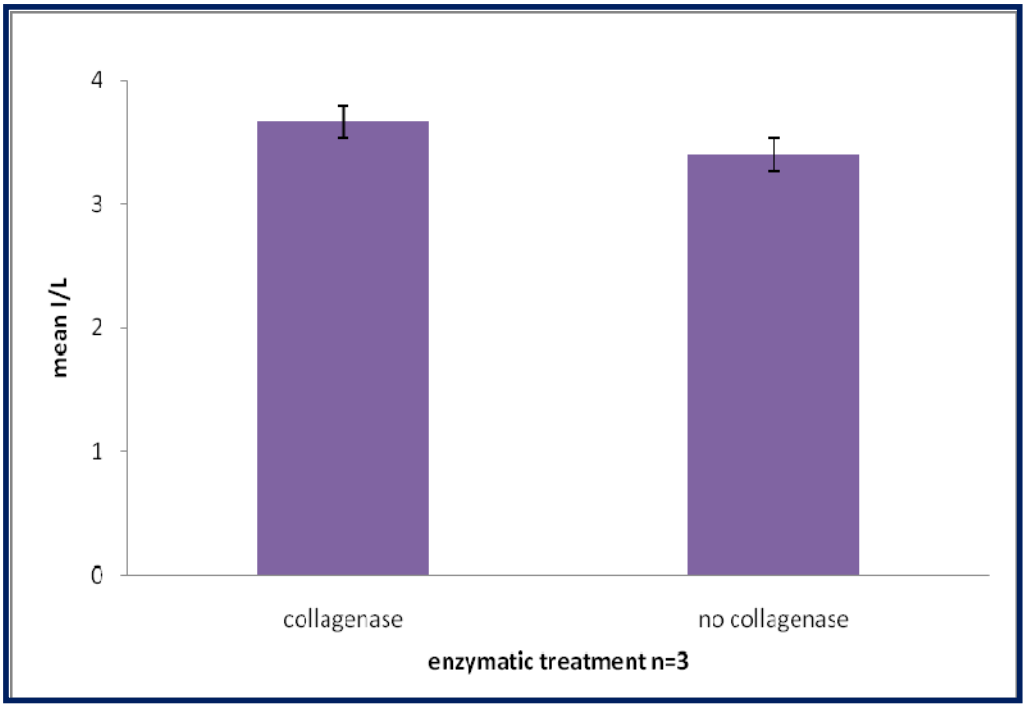

4
3
2
1
0
mean l/L
collagenase
no collagenase
enzymatic treatment n=3

Figure 3.7: A comparison on adult zebrafish neurite growth in the presence and absence of collagenase at 28.5º C.

Adult zebrafish neurons extending neurites in BSF2 medium in the presence and absence of collagenase. The extent of neurite growth were quantified by using a test grid analysis as described in the general methods section . In this histogram, the vertical axis represents the number of intersection of the test lines with neurites (I/L) and is a measure of the extent of neurite arborisation (see General Methods). Each condition was investigated six times and each bar gives the mean = SEM of the number of intersection per unit test line applied. This histogram shows that there was no significant positive or negative effect on neurite growth of incubating adult zebrafish CNS neurons in collagenase for 30 minutes compared to neurons grown without such enzymatic treatment. In all cases, the neurons were grown in the incubator at a temperature of 28.5˚C with the atmosphere containing 1.5% CO_2 for a period of a week.

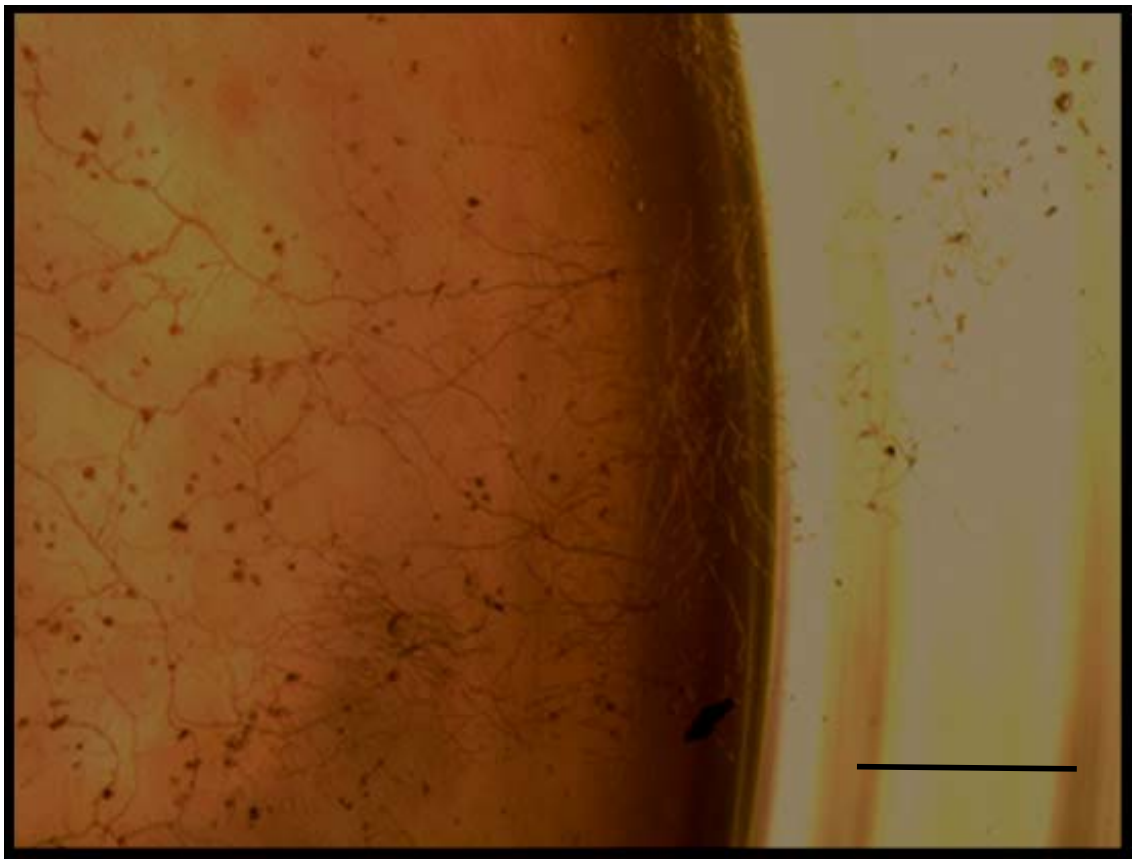


Figure 3.8: Growth of dissociated adult zebrafish neurons on coverslips after 14 days.

Phase contrast image of adult zebrafish neurons extending neurites in BSF2 medium coated with poly-L-lysine coverslip. The photgraph was taken after fourteen days of incubation at 28.5˚C with the atmosphere containing 1.5% CO_2. This micrograph shows a dense cluster of neurons extending their neurites (arrows) towards the edge of a coverslip. (Scale bar=50μm).

Table 3.1: A summary of the results of different trials under varying conditions designed to examine the optimal conditions in which to grow adult zebrafish CNS neurons.

Formulations	Temperature	Atmospheric CO_2	Media	Enzymatic treatment	Result
Trial 1	37.0ºC	5.0%	BSF2	collagenase	No neurite growth
Trial 2	28.5ºC	1.5%	L-15	collagenase	7days-Small neurites appear 14 days- small, short neurites extending from the cell body
Trial 3	28.5ºC	1.5%	L-15	Without collagenase	7days-Small neurites appear 14 days- small, short neurites extending from the cell body
Trial 4	28.5ºC	1.5%	BSF2	Without collagenase	7days-Small neurites appear 14 days- small, short neurites extending from the cell body
Trial 5	28.5ºC	1.5%	BSF2	collagenase	7days-Long braches of neurites appear 14 days-extensive neurite growth shown extending from the cell body– **BEST COMBINATIONS**

3.4 DISCUSSION

The major result of this study was the demonstration that isolated neurons from the adult zebrafish brain were capable of surviving and regenerating their neurites in a tissue culture system. This is the first study to thoroughly demonstrate the most suitable and optimal conditions for growing adult zebrafish neurons (Table 3.1 and Figure 3.2). It was also noted that there were several previous studies (Kuwada et al., 1990, Hanna et al., 1998, Becker and Becker, 2002, Nusetti et al., 2006, Yanicostas et al., 2009, Kanungo et al., 2011, Sugitani et al., 2012) that had evaluated the axonal regeneration in lower vertebrate species such as goldfish or other teleost species. However, these previous studies used embryonic stages of development of certain parts of the brain (retinal ganglion cells and olfactory system) as the source of the neurons they used. Many recent studies(Bastmeyer et al., 1993, Becker and Becker, 2002, Brosamle and Halpern, 2002, Buckley et al., 2008, McCurley and Callard, 2010, Raphael and Talbot, 2011)) have investigated the adult zebrafish whole brain using gene chip microarray analysis in order to determine factors that may enhance axonal regeneration in these species. However these studies did not require zebrafish neurons to be grown in culture.

In the present study, the main objective was to develop the most effective and convenient method for isolating and culturing adult zebrafish (*Danio ranio*) CNS neurons to obtain maximum neurite outgrowth. From the result obtained in this experiment, it was noticeably evident that adult zebrafish neurons could survive longer than a week in the tissue culture system and the neurites were visibly branched out into a network. Recent work by (Tomizawa et al., 2001) revealed that adult zebrafish whole brain could be organ-cultured in an ex vivo system. The study

mainly assessed the tissue organization and morphology of the brain as well as cell viability and survival by examining the uptake of horseradish peroxidase (HRP). However, it was found that such whole brain explants were not capable of surviving for more than about seven days in such culture. Study by Tomizawa (2001) did not attempt to isolate individual zebrafish neurons in order to study their growth and regeneration properties.

In my study, adult zebrafish brain was used instead of immature or juvenile brains. The regenerative capacity of neurons from immature versus mature brains is known to be markedly different in mammals. It is therefore likely that the same would be true of zebrafish. In addition, the adult brain is characterized by a marked increase in size and complexity. The neurons in the mature brain are much more intricate with a complex dendritic network and fully developed axons than those obtained from immature brains. Adult zebrafish brains are also known contain numerous other cell types (microglia, oligodendrocytes, and astrocytes) that are also present in adult mammalian CNS. These factors make the adult zebrafish brain a more useful model to study than the immature zebrafish brain when the purpose is to examine the factors which allow neuronal and axonal regeneration after injury (Cho et al., 2007, Kizil et al., 2012). Despite this, *in vitro* studies (Mahler and Driever, 2007, Chen et al., 2013) on the zebrafish brain to date have used cultures obtained from immature brains. It seems that this has been so because of a lack of methods described in the literature which allow neuronal cultures to be obtained from the adult zebrafish brain. I have now developed such a method as reported in this chapter.

The development of a tissue culture system to examine neurite growth from adult zebrafish CNS neurons was an essential pre-requisite step without which the rest of the studies reported in this thesis could not have been completed. It is known that the growth media is a source of nutrients and is not the only factor that facilitates the cell growth effectively, but other environmental factors also have a significant influence on neurite outgrowth, differentiation and apoptosis. (Singh et al., 2008) Different cells require different conditions to stimulate their growth (Georges et al., 2006). Various adjustments of the environmental factors and nutrients were assessed in order to determine the optimal conditions for growing adult zebrafish CNS neurons. Combinations of media, temperature, CO_2 levels and the presence or absence of enzymatic treatment were altered till the optimal conditions had been determined. Different trials have been summarised in table 3.1

My findings indicated that the adult zebrafish neurons grow best in BSF2 medium at the temperature of 28.5ºC with an atmosphere of 1.5% CO_2. The presence of collagenase had no significant effect on neurite growth compared to the untreated condition.

The adult zebrafish brain cells were cultured and dissociated in two kinds of media; BSF2, which is known for culturing most mammalian neuronal cells and L-15, which is specifically used for lower vertebrates species (Johnson and Turner, 1982, Connaughton and Dowling, 1998, Cormie and Robinson, 2007). It was evident that both media permit the growth of adult zebrafish cultured neurons after a few days of incubation. There was no neurite sprouting shown in the first few days after dissociation and culturing of the cells with either of the media. The cells were left undisturbed in the incubator to allow the brain cells to adhere to the well-plate or

coverslips. It was notable that adult zebrafish neurons commenced regeneration of neurites after seven days of culturing under the adjusted conditions in both supplemented media. The neurites extended their branches out from the cell bodies extensively with BSF2 and other adjusted environmental conditions after fourteen days; further experiments were performed after this period such as immunohistochemistry staining with GAP-43 antibody.

Unlike most of the neural cells, adult zebrafish cells are relatively simple to dissociate and grow when the right conditions are maintained in a humidifying incubator (Further details are mentioned below and in method section). These types of neurons seemed to a take a longer time to grow and extend their neurites compared to rat DRG neurons. Other studies have revealed that rat DRG cells commence their neurite growth between about 2 to 4 days after being place in tissue culture but could only maintain such growth for approximately a week before the culture became degraded (Bedi et al., 1992, Pazyra-Murphy and Segal, 2008). On the other hand, this investigation showed that adult zebrafish neurons could be kept in culture for up to about three weeks before the cultures showed signs of deterioration. The exact reason for this are unknown but may be related to the effective period of the tissue culture medium and the active period of antibiotic components. Whether or not cultures could be maintained for longer than three weeks by replacing or replenishing the tissue culture medium is unknown as such experiments were not attempted. Most cell lines, including mammalian DRG neurons, require an enzymatic digestion in order to dissociate and grow the neuronal cells productively (Bedi et al., 1992). Interestingly, collagen treatment to dissociate the cell is not necessary when working with lower vertebrates. Collagenase is the

enzyme that breaks the peptide bonds in collagen. Since there is no collagen present in the extracellular matrix of the cell, this enzymatic treatment is not essential in maximising the growth of adult zebrafish neurons.

Cell culture conditions vary broadly for different cell types, but the artificial environment within the incubator in which the cells are cultured and grown generally contains suitable medium, growth factors, gases (oxygen and carbon dioxide) and physiological regulators for factors such as pressure, pH and temperature. The medium used for cell culture control and balance the pH of the culture. The pH of the medium is dependent on the balance of dissolved carbon dioxide and bicarbonate, changes in the atmospheric CO_2 can changed the pH of the medium. This makes it essential to use exogenous CO_2 to control and prevent cell contamination and apoptosis. In the mammalian cell line, the most suitable atmospheric CO_2 within the incubator has been found to be around 5.0% with the optimal temperature of 37ºC. Some species require a smaller amount of carbon dioxide gas and a lower temperature in order to survive and grow. Due to the lack of studies describing the exact methods for growing adult zebrafish neurons, many attempts to develop this method were unsuccessful in the first three to six months of this project. The main concern was the temperature and the atmosphere inside the incubator. Initially, the cells were grown at a temperature of 37˚C with 5% CO_2, the optimal conditions for growth of other mammalian cell-lines. (Lemmon et al., 1992, Wilson et al., 2000). Under such conditions, however, it was found that there were no growths of neurites although the dissociated neuronal cells appeared to remain alive. The body temperature of cold-blooded zebrafish is much lower than warm-blooded mammals; therefore, it was considered that the incubation temperature of neurons derived from

zebrafish may well be different to that normally used for culturing mammalian neurons. Growing adult zebrafish neurons at an incubation temperature normally used for mammalian cells would likely cause an imbalance of protein synthesis and denaturation and result in cell apoptosis. During development, it has been suggested the zebrafish neurons grow best at 27˚C-28.5ºC with a lower percentage of CO_2 (Tomizawa et al., 2001, Myhre and Pilgrim, 2010). In this study, the temperature of the incubator was adjusted to 28.5ºC as the fish were kept in the tank with that temperature prior to conducting any experiments (Tomizawa et al., 2001). Furthermore, atmospheric carbon dioxide level is known to have a great impact on growing neurons. It is critical to maintain the level of CO_2 in the incubator at the concentration corresponding to the buffer used in the tissue culture medium and of a similar value as the pH of the cells are being studied (Qin et al., 2010). The physiological blood pH of the adult zebrafish in water is approximately 7.8 (Lawrence, 2007). Thus, it is compulsory to adjust the CO_2 levels to the right percentage in the incubator for cell regulation and survival (Qin et al., 2010). After adjusting all these environmental factors, this has become the novel method that may allow the growth of adult zebrafish neurons to develop most efficiently.

As mentioned earlier in chapter 1, it is not easy to distinguish between axons and dendrites in tissue culture on morphological criteria alone. Indeed, this is the reason why we refer to elongated cell processes as neurites when discussing cultured neuronal cells. It has been suggested by Matus (1988) that in mammalian cells, microtubule-associated proteins (MAP) may be used to distinguish between axons and dendrites (Matus, 1988). It has been suggested that MAP1 and MAP2 may be specific markers for axons and dendrites respectively (Wiche et al., 1983, Chamak et

al., 1987, Lewis et al., 1988, Lewis et al., 1989). If this is also true for zebrafish neurons it may be possible to identify given neurites as axons or dendrites although such experiments were not attempted during the present investigation. However, studies by Matus and Hirokawa's group focused on the distribution of MAP in the rat brain using monoclonal antibodies. The immunohistochemistry staining revealed that both MAP1 and MAP2 are present only in neurons and both are highly concentrated in dendrites compared to axons. Otherwise, they show different distribution in several ways. MAP1 was detected at low levels in axons, whereas MAP2 was not visible in axons with either of two different fixation methods used (Matus et al., 1986, Hirokawa et al., 1988a, Hirokawa et al., 1988b, Sato-Yoshitake et al., 1989). Due to the fact that MAP1 is detectable in dendritic network, this methodology remains controversial and not fully validated. Further investigations and examinations related to quantification of neuronal outgrowth may also be required. In this study, the whole brain was dissociated and grown under tissue culture conditions. The next step might be to examine separate but defined parts of the brain and spinal cord to determine whether the neurons from specific regions are also capable of neurite growth. This would allow us to gain further insight into how neurons in each part of the zebrafish CNS grow and how this may be important for neuronal regeneration.

It is evident from the present study that adult zebrafish neurons are capable of extending their neurites in a tissue culture system. It may also be important to determine whether these neuronal cells are also capable of proliferating within a tissue culture system. Whilst such a study has not been attempted during the present project due to limited time available, it may best be determined using a bromodeoxyuridine (BrdU) labelling assay (Zupanc et al., 2005). BrdU is widely used

to label proliferating cells during animal development. BrdU is a thymidine analog that binds to DNA of the dividing cells during S-phase of the cell cycle. It is a marker for DNA synthesis and often used for monitoring cell proliferation especially for neuronal cells. BrDU labelled cells can be subsequently identified by immunocytochemical techniques using an anti-BrdU antibody and immunochemistry by using antibody labelled against a single stranded DNA that contains BrdU (Ghosh et al., 1997, Kishimoto et al., 2012).

CHAPTER FOUR

METHODS TO ISOLATE AND GROW ZEBRAFISH CNS NEURONS IN TISSUE CULTURE: EFFECTS OF VARIOUS NEUROTROPHIC FACTORS

4.1 INTRODUCTION

Neurotrophic factors (NTFs) are endogenous molecules which play an essential function in the maintenance, survival and differentiation of different neuronal populations during the development of adult brain (Henderson, 1996, McAllister et al., 1999, Schutte et al., 2000). The properties of NTFs have been investigated in both *in vitro* and *in vivo* experimental models of brain injury (Lindsay, 1988, Liu and Snider, 2001, Markus et al., 2002, Logan et al., 2006). NTFs have been shown to promote neuronal cell survival and enhance axonal regeneration as well as neuronal plasticity (Miyata and Yasuda, 1988, Huang and Reichardt, 2001). The numerous essential attributes of NTFs highlights the need to investigate their use as therapeutic approaches for treating secondary damage after brain injury.

Research had established that environmental conditions existing within the CNS can greatly influence any regenerative processes (Riley et al., 2004). The presence or absence of suitable neurotrophic factors at the injury site can govern the extent of neuronal survival and any axonal regeneration. Furthermore, as mentioned previously, there are a number of inhibitory factors present in the post-lesion CNS that limit or restrict the extent of any regenerative processes (Mckerracher et al., 1994, Mukhopadhyay et al., 1994, Niederost et al., 1999, Liu et al., 2002, Wang et al., 2002).

One of the therapeutic approaches in promoting and supporting axonal regrowth is the administration of neurotrophic factors to the injured area within the CNS (Kawaja and Gage, 1991, Stichel and Muller, 1998). Recently, various neurotrophic factors were tested in many studies involving regeneration of nerve cells (Mcfarlane et al.,

1995, Shen et al., 1999, Goldberg et al., 2002, Deister and Schmidt, 2006). For instance, in the visual system, regeneration of retinal ganglion cells (RGCs) were investigated using a crushed model of the optic nerve, where the integrity of the nerve is conserved and stabilised, but the transfected axons face the non- conducive environment (Berry et al., 1996). It has been found that the application of neurotrophic factors could enhance both the survival of RGCs after the crush, as well as promote the regrowth of axons into the optic nerve (Mansourrobaey et al., 1994, Lorber et al., 2005, Logan et al., 2006).

Moreover, studies on combinations of several neurotrophic factors have been carried out to examine whether the response to regeneration is more effective compared to the application of a single growth factor (Goldberg et al., 2002, Deister and Schmidt, 2006). The study by (Mansourrobaey et al., 1994) also illustrated that the administration of combinations of neurotrophic factors, FGF-2, NT-3 and NGF caused a synergistic response on RGCs axonal regeneration after optic nerve crush (Sapieha et al., 2003, Logan et al., 2006). It was evident that the combination of treatments provided a more effective result in promoting the regrowth of axons than treatment with a single growth factor (Goldberg et al., 2002, Logan et al., 2006). This may be because different neurotrophic factors promote growth in different types of nerve cells.

In addition, it was shown that BDNF and FGF-2 could enhance the regeneration of spiral ganglion cell axons into the injured organ of Corti (Leake et al., 2011). BDNF and NT-3 encouraged corticospinal projection and propriospinal axonal regeneration in the adult rat spinal cord (Xu et al., 1995). These studies suggested that each individual neurotrophic factor treatment had a specific therapeutic effect on different

parts of the axon, but in general, they allowed axon regeneration in the unfavourable CNS environment.

In contrast to mammalian CNS, the nervous systems of teleost fish and amphibians have a vigorous regeneration capacity after traumatic injury. It is thought that they are well- characterised by their ability to produce new neurons in the adult CNS. This ability allows such animals to replace lost neurons with newly generated neurons after lesion (Sperry, 1963). This phenomenon is, unfortunately, largely unavailable in the adult mammalian CNS. In the teleost fish brain, the new neurons are generated in large quantities in different proliferation zones. It has been implied that the proliferation activity can be regulated by several molecular factors to significantly enhance regeneration of neurons (Zupanc, 2006b).

Goldfish or zebrafish can regrow their axons after the optic nerve transection to re-innervate and restore vision (Mizrachi et al., 1986, Bastmeyer et al., 1993). Similar to mammals, neurotrophic factors and proteins secreted by the sheathing cells of the optic nerve known as axogenesis 1 and 2 also promote and stimulate the outgrowth of explanted fish RGCs *in vitro* (Schwalb et al., 1995, Benowitz et al., 1998, Jo et al., 1999). Myelin-based inhibitors are also present in fish optic nerve, but the composition and the signalling pathway seemed to differ from mammalian CNS (Sivron et al., 1994). This might be the reason why RGCs explanted from fish promotes the regeneration of myelin more vigorously and seem to be less sensitive to myelin inhibitory molecules while the regeneration of rat myelin is not possible (appendix 10).

Another study (Lucini et al., 2007, Lucini et al., 2010) demonstrated that the Glial cell line-derived neurotrophic factor (GDNF) is an important growth factor expressed in many neuronal populations in both the CNS and PNS of both mammalian and lower vertebrates. It is also well-characterised in lower vertebrate species such as the zebrafish brain. The results from the study suggested that the expression of GDNF is not only high during the developmental period, but is also highly expressed in adult zebrafish retina and contributes to retinal regeneration (Nosrat et al., 1996, Hitchcock et al., 2004, Lucini et al., 2007).

Neurotrophins are proteins that are known to regulate neuronal survival, axonal and dendritic growth, and synaptic plasticity during development. It has been suggested that these factors may allow the adult mammalian CNS neurons to survive and to regenerate cell processes after injury. Thus, a greater understanding of how neurotrophic factors can enhance axonal regeneration in both mammalian and lower vertebrates is required to identify future treatment strategies for adult mammalian CNS after traumatic lesion. In this study, several neurotrophic factors were applied to adult zebrafish neurons in the tissue culture system. This experiment aimed to examine whether neurotrophic factors increase the growth of the neurites and which is the best or the most sensitive to adult zebrafish CNS neurons. It was hoped that the results from these experiments will provide a clearer view of the possible role of how various growth factors help in promoting and maintaining axonal growth.

4.2 METHODS

4.2a) Application of neurotrophic factors to the growing adult zebrafish neurons

Neurons from adult zebrafish brain were isolated and grown in tissue culture, as described earlier in chapter 3, in the presence of various neurotrophic factors. Each neurotrophic factor examined was used at two different concentrations designated as high (H) and low (L). The concentrations used for these factors were adapted from the Sigma's company St. Louis, MO product user instructions. Low (1µl/ml) and high concentrations (5µl/ml) of each neurotrophic factors were added to neuronal cell tissue culture dishes and were incubated at 28.5^{o}C in an atmosphere of 1.5% CO_2 for fourteen days. Six separate neuronal tissue cultures, each derived from separate zebrafish brains, were examined for each neurotrophic factor at each of the high and low concentrations.

The Neurotrophic factors that were used in these experiments were:

1. **Nerve growth factor (NGF)** (Sigma St. Louis, MO)
2. **Brain-derived neurotrophic factor (BDNF)** (Sigma St. Louis, MO)
3. **Neurotrophin-3 (NT-3)** (Sigma St. Louis, MO)
4. **Leukemia growth factor (LIF)** (Sigma St. Louis, MO)

4.2b) Method of quantifying the neurite growth of adult zebrafish

After fourteen days of culture, the extent of neurite growth was quantitatively assessed for each preparation using a 1cm test grid analysis, as described earlier in chapter 2.4. Differences between control and treated groups were examined statistically using either t-tests or one-way ANOVA procedures.

4.3 RESULTS

ADULT ZEBRAFISH NEURONS IN THE PRESENCE OF NEUROTROPHIC FACTORS

Table 4.1: The effect on four neurotrophic factors on the neurite growth of adult zebrafish

Concentrations	Control	NGF	LIF	BDNF	NT-3
Low (1µl/ml)	+	++	+++	+++	+++
High(5 µl/ml)	+	+++	++++	+++	++

The table provides a qualitative measure of the effect on each neurotrophic factor at both low and high concentrations. The numbers of the + symbols in any given condition indicates an increase in the degree of neurite extension as assessed by simple qualitative observations. It can be seen by that the administration of LIF at high concentration appeared to have had the greatest effect on neurite growth in the adult zebrafish in the tissue culture system. BDNF and NGF had equal effects in producing growth. Furthermore, NT-3 also caused an increase in neurite growth when compared to the control. In order to be certain and confirm which neurotrophic factor had the greatest effect in enhancing the neurite growth of adult zebrafish, a quantitative analysis of the neurite growth under the various treatment conditions was carried out using a test grid system as described in chapter 2.

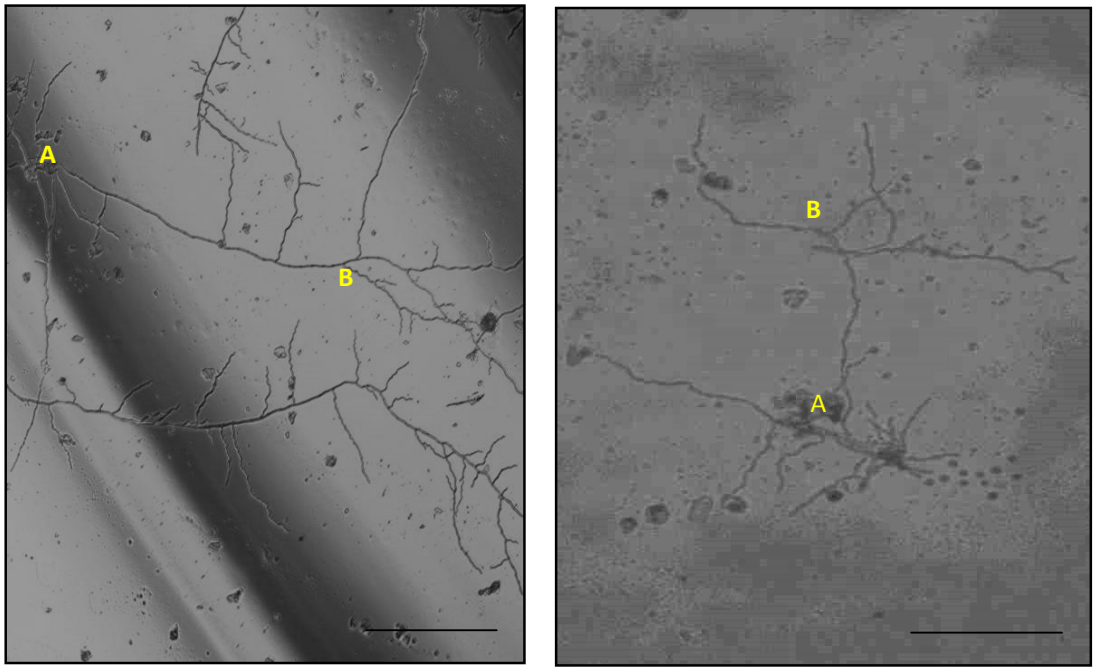

A
B
B
A

Figure 4.1: Micrographs of adult zebrafish neurons in the presence of neurotrophic factors.

These micrographs show example of adult zebrafish neurons images growing after fourteen days in the presence of neurotrophic factor (LIF) at high concentration and the control. The administration of neurotrophic factor did enhance the neurite growth significantly compared to the control culture that was conducted in chapter 3. A= cell bodies ; B= neurites (scale bar = 50μm).

4.3a) The effect of neurotrophic factors in promoting adult zebrafish neurite outgrowth

The extent of neurite extension in the presence of various neurotrophic factors are shown in figure 4.2. In this, it can be seen that a high concentration of LIF resulted in there being about 14 intersections per unit test line with extending neurites. This compared to a figure of about 6 intersections per unit test line under the control condition when there was an absence of any neurotrophic factors. A low concentration of LIF also resulted in an increased amount of neurite extension compared to the control condition, although this was lower than the high concentration micrograph (Figure 4.2). Thus, under this condition, there were about 9 intersections per unit test line applied. It is also seen that NT-3 has a considerable effect on promoting the neurite growth. At low concentration, the extent of neurite growth resulted in about 8 intersections per unit test compared to about 6 for the control treatment. The lower concentration of NT-3 did not have much influence on the growth compared to the higher dosage treatment. The result of high concentration of NT-3 in the neurite growth was approximately 9 intersections. The increase in the neurite growth using this neurotrophic factor was dosage-dependent. Similarly, in the presence of BDNF in the high concentration condition, the neurite growth was observed to be significant, about 8 intersections per unit sample compared to about 6 intersections of the control treatment. The low concentration of NGF resulted in minimal neurite extension relative to the control, about 7 intersections per unit test line. However, at high concentrations, there was significant growth, as seen in Figure 4.2 with about 10 intersections per unit test line compared to the control of about 6 intersections per unit test line.

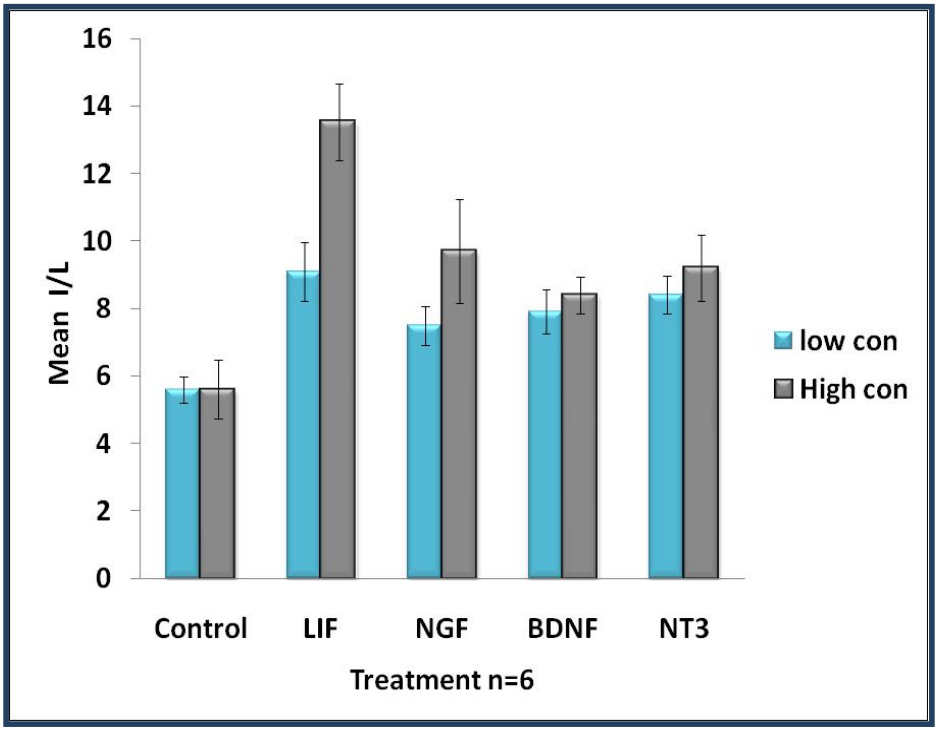

16
14
12
10
8
6
4
2
0
Mean I/L
Control
LIF
NGF
BDNF
NT3
Treatment n=6
low con
High con

Figure 4.2: The effect of four neurotrophic factors on neurite extension of adult zebrafish.

The extent of neurite growth in adult zebrafish neurons grown in tissue culture for a period of fourteen days in the presence of two different concentrations of four neurotrophic factors is illustrated above. All neurotrophic factors appeared to be significant compared to the control condition. However, LIF at both low and high concentration were found to have the greatest effect.

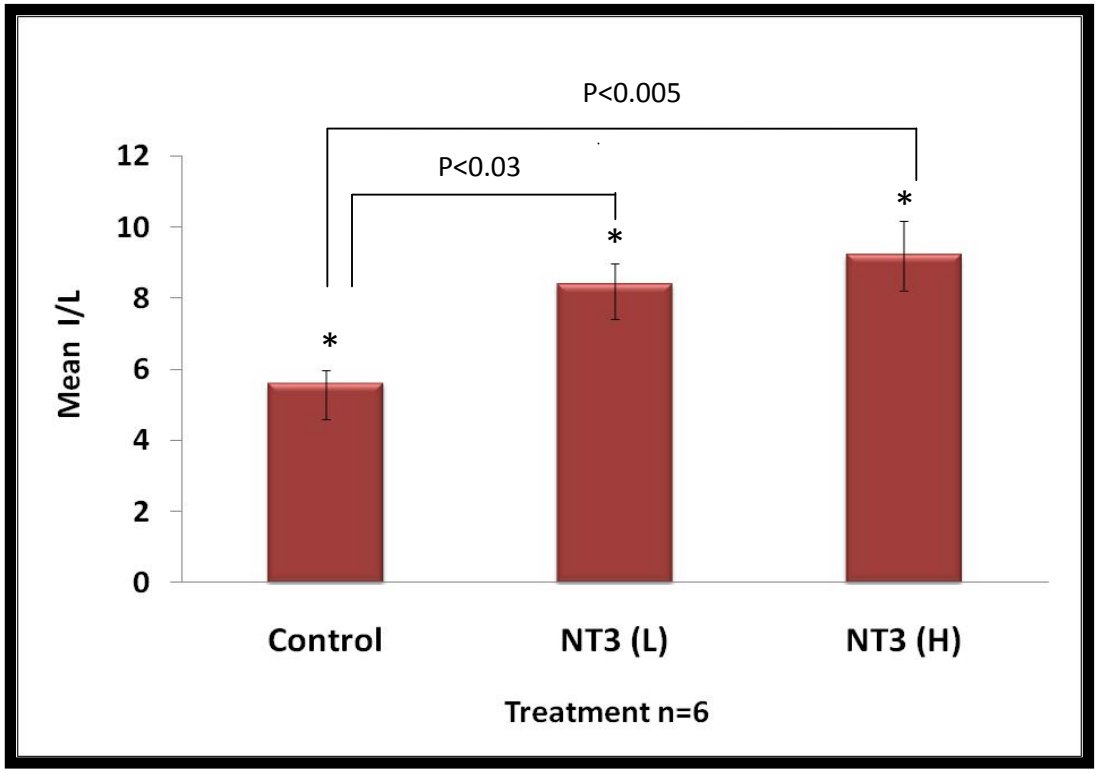

P<0.005
P<0.03
12
10
8
6
4
2
0
Mean I/L
*
*
*
Control
NT3 (L)
NT3 (H)
Treatment n=6

Figure 4.3: The effect of neurite growth in the presence of low and high concentrations of neurotrophin-3 (NT-3).

The histogram demonstrated that NT-3 at both low and high concentration caused a significant increase in the extent of neurite growth compared to the control. There was no significant effect between NT-3 (L) and NT-3 (H). (F value F=7.61; P value=P<0.005)

*p<0.05 compared to the control group

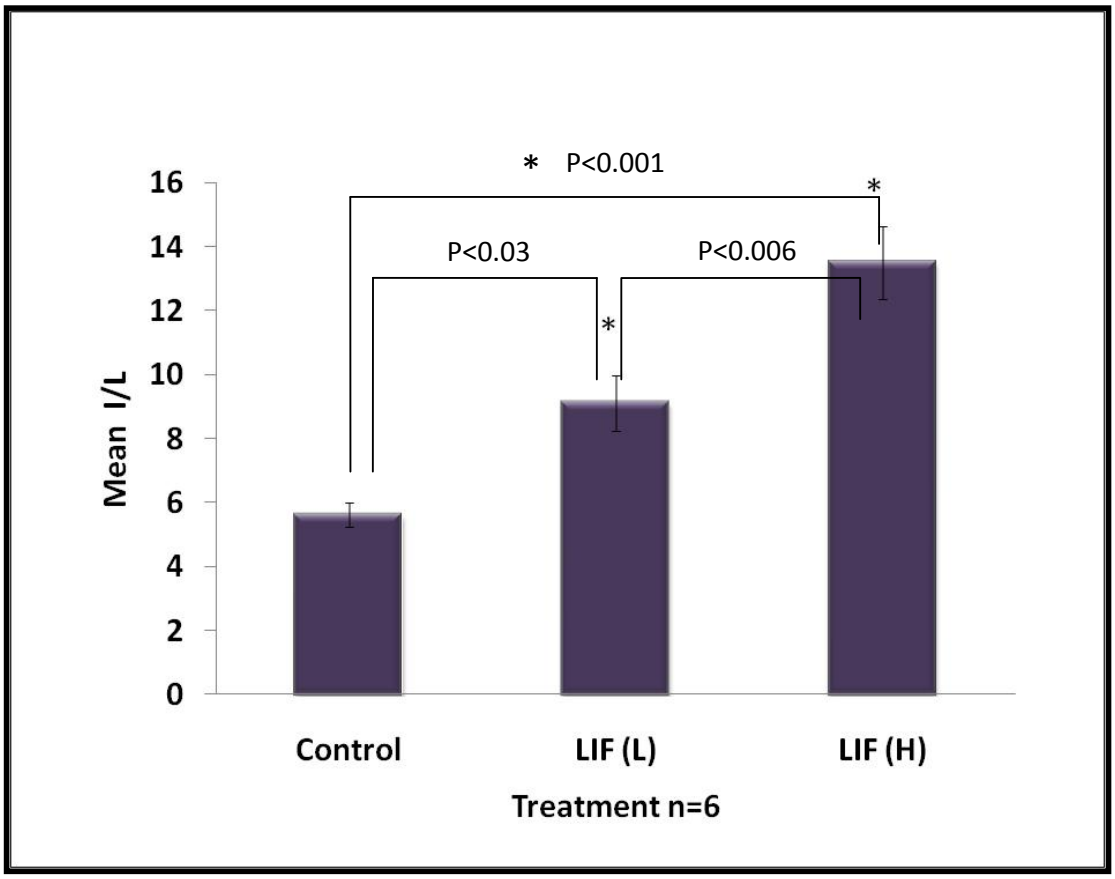

* P<0.001
*
P<0.03
P<0.006
*
16
14
12
10
8
6
4
2
0
Mean I/L
Control
LIF (L)
LIF (H)
Treatment n=6

Figure 4.4: The effect of neurite growth in the presence of low and high concentrations of leukemia inhibitory factor (LIF).

The histogram illustrated that LIF at both low and high concentration caused a significant increase in the extent of neurite growth compared to controls. The effect was greater at the higher concentration used. The number of intersections between the dendrites and the grid were counted and expressed as the number of interstections per unit length of test line (mean I/L). (F value F=21.62; P value=P<0.001)

*p<0.05 compared to the control and low dosage of drug

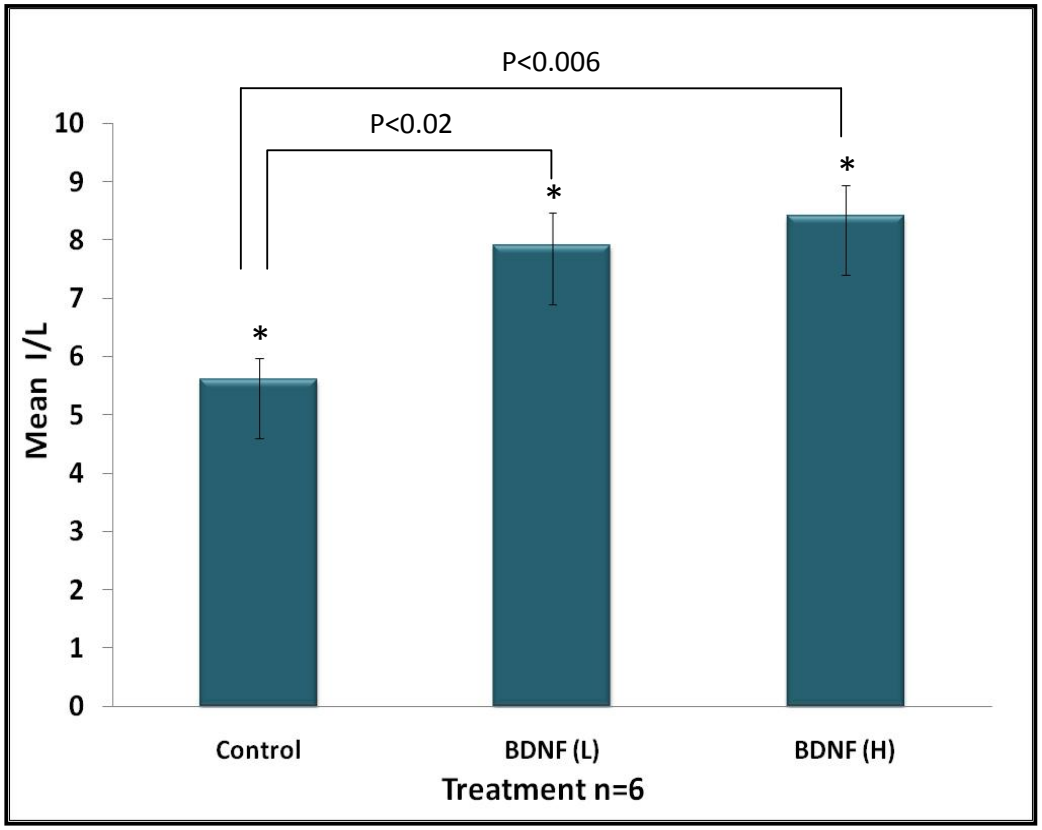

P<0.006
P<0.02
*
*
*
10
9
8
7
6
5
4
3
2
1
0
Mean l/L
Control
BDNF (L)
BDNF (H)
Treatment n=6

Figure 4.5: The effect of neurite growth in the presence of low and high concentrations of Brain Derived neurotrophic Factor (BDNF).

The histogram demonstrated that BDNF at both low and high concentration caused a significant increase in the extent of neurite growth compared to the control. However, there was no significant outgrowth increase between low and high concentration of BDNF. The number of intersections between the dendrites and grids were counted and expressed as the number of interstections per unit length of test line (mean I/L). (F value F=7.75; P value=P<0.005)

*p<0.05 compared to the control group

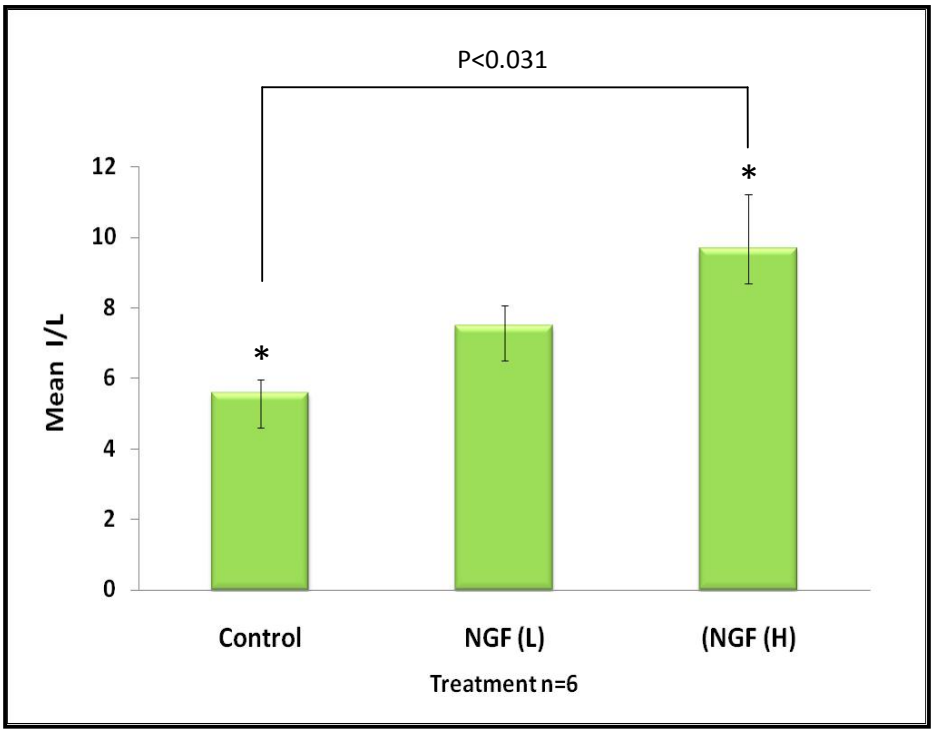

P<0.031
12
10
8
6
4
2
0
Mean I/L
*
*
Control
NGF (L)
(NGF (H)
Treatment n=6

Figure 4.6: The effect of neurite growth in the presence of low and high concentrations of Nerve Growth Factor (NGF).

NGF at low concentration caused a slight increase of adult zebrafish neurite growth compared to controls. NGF at high concentration had a significant effect compared to the control (F value F=4.43; P value=P<0.031). However, there was no significant difference in neurite extension between low and high concentration used.

*p<0.05 compared to the control group

4.4 DISCUSSION

The main findings of this study directly supported the hypothesis that neurotrophic factors have a vital role in improving neurite regeneration in adult zebrafish neurons in the cell culture system. To date, abundant neurotrophic factors have been identified and widely used in neuro-regeneration studies either in neuronal cell lines *in vitro* or *in vivo*. The choice of neurotrophic factors used in various studies is mainly dependent upon the neuronal cell types, as different parts of CNS are sensitive to certain growth factors.

Each neurotrophic factor acted as a growth factor that mediated the regeneration, differentiation and survival of neurites by specific signals and receptors within the CNS. To understand the underlying mechanisms of regeneration, we have used *in vitro* methods to culture adult zebrafish in the presence of neurotrophic factors. The experiments performed in this study involved administration of four neurotrophic factors in the dissociated adult zebrafish neurons *in vitro*

These four neurotrophic factors (NGF, NT-3, BDNF and LIF) were chosen based on previous studies carried out by other groups (Namiki et al., 2000, Goldberg et al., 2002, Sharma, 2007).

Many studies have suggested that NGF increased the expression of neuronal growth (Schwab et al., 1979). Alternatively, different members like NT-3 and BDNF were also used broadly in many studies (Namiki et al., 2000). The effect of LIF on the enhancement of neurite growth in lower vertebrates, on the other hand, has not been observed closely by many studies (Cafferty et al., 2001, Teng and Tang, 2006). Overall, this experiment was conducted to measure the level of neurite outgrowth in

response to growth factor administration and which treatment would be the most effective and sensitive to adult zebrafish neurons.

The observations in this study have shown that these four neurotrophic factors contributed greatly in enhancing neuronal outgrowth in both low and high concentrations *in vitro*. Interestingly, it was demonstrated that LIF at high concentrations appears to give the best response when compared to the other three treatments.

Generally after lesion, axons would have to overcome the scar barrier in order to regenerate. During this phase, the axons would face the obstacle of choosing the correct pathway to their former target sites that allow the neurons to regrow and extend (Davies et al., 1999). One possible approach to develop the outgrowth of neurite is through secretion or administration of growth factors within the CNS. These growth factors might be secreted from denervated targets and can attract regrowing neurons to the right connection and locations. (Cai et al., 1999).

Varieties of neurotrophic factors have been investigated intensively in higher vertebrate species (Lentz et al., 1999, Cafferty et al., 2001) . For instance, in DRG neurons, it has been implied that growth factors can strongly potentiate neuronal outgrowth especially when combining two growth factors together. A study by (Lindsay et al., 1985, Lindsay, 1988) indicated that the use of NGF and BDNF, either singly or in combination, can induce the growth of axons drastically but were not mandatory for survival of adult sensory neurons. Recent studies by (Barde et al., 1982, Marshak et al., 2007, Numakawa et al., 2010, Panagiotaki et al., 2010) have evaluated the role of BDNF in the Trk B signaling pathway in adult zebrafish neurons. It was proposed that application of BDNF treatment could potentially

influence the neurite regeneration (Huang and Reichardt, 2001, Song et al., 2008, Liu et al., 2011). Similarly, the results of this present study have verified that BDNF has an influence in promoting neurite regeneration by branching out and forming the arborisation.

A study by Hanington's group focused on the function of LIF on both embryonic and adult zebrafish. LIF belongs to a member of Interleukin- 6 (IL-6) cytokine family which is responsible for the maintenance, survival and the repairing of neuronal populations (Hanington et al., 2008). The role of LIF has been well-observed in mammals in past decades, however, few groups have speculated that LIF may stimulate neurite regeneration in zebrafish (Bauer et al., 2003, Hanington and Belosevic, 2005, 2007, Hanington et al., 2008, Deverman and Patterson, 2009). The study was conducted to examine the expression of molecules in adult zebrafish. It was noted that LIF was highly expressed in the brain and kidney (Hanington et al., 2008). The positive role of LIF has been observed in neuronal development and survival not only in mammals, but also in teleosts. The result of this study is consistent with previous reports suggesting that LIF increased the neurite outgrowth extensively within a fourteen day period of incubation. Adult zebrafish neurons were clearly responsive to LIF treatment and that specific receptors and signaling pathway regulated the neurotrophic factors. The signals from growth factors must be transmitted sufficiently to the neurons to maintain their survival and growth and overcome the interference within the CNS after injury. The results demonstrated that different neurons were commonly responsive to different growth factors and multiple factors (adhesive molecules) could be combined to stimulate a greater effect in neurite growth. In summary, it is now confirmed that neurotrophic factors are

significant in increasing the neuronal outgrowth, specifically treatment with LIF on adult zebrafish neurons.

In future studies, more neurotrophic factors such as ciliary neurotrophic factor (CNTF) (belonging to the same family as LIF), GNDF and FGFs should be used to investigate whether the production or presence of these neurotrophic factors could have a greater effect in increasing neurite growth compared to LIF. Combinations of neurotrophic factor, for instance, (LIF +BDNF) may be investigated to examine their effect in increasing neurite outgrowth. It is also necessary to try out different doses of each neurotrophic factor used in this study to determine a dose dependent curve. This would allow us to identify the exact concentration of neurotrophic factor that provides the best growth for the neurite. This preliminary work may also lay the foundation for further studies in the potential therapeutic role of these neurotrophic factors in improving functional regeneration.

CHAPTER FIVE

THE EFFECT OF SUBSTRATES ON ATTACHMENT AND GROWTH OF CULTURED ADULT ZEBRAFISH NEURONS

5.1 INTRODUCTION

The nervous system is a complex intricate network where communication between neuronal cells and their surroundings result in the transmission of information which generates reactions between the internal cells and the environment (Hollyday, 1983, Herculano-Houzel, 2011, Schoenemann, 2012). Neurons are known to be processors and essential transmitters that relay information throughout the whole nervous system. Importantly, the generation, maintenance and processing of networks are dependent on the development and function of the neurons (Raff et al., 1993, Stuart et al., 1997). As neurons grow, cell extensions axons and dendritic branches develop; these branches interact with other neurons establishing appropriate networks (Lnenicka and Atwood, 1985, Lee and Luo, 1999, Tang et al., 2001, Da Silva and Dotti, 2002, Jarrell et al., 2012). Neurons are versatile cells with highly developed path-finding abilities during their development (Brustle, 1999, Araque et al., 2001, Lagercrantz and Ringstedt, 2001, Ziemba et al., 2008). However, the environmental conditions are quite different during the developmental stages compared to adult life after injury.

Neurons are endowed with varieties of mechanisms that allow them to integrate within their neurite networks. This includes extra-cellular matrix (ECM) (Faissner et al., 2010, Frischknecht and Gundelfinger, 2012). The ECM is comprised of several extracellular adhesion molecules such as fibronectin, laminin or collagen which allow cells to adhere to the ECM (Akers et al., 1981, Bixby and Jhabvala, 1990, Sheppard et al., 1991, Miner and Yurchenco, 2004, Ahmed et al., 2005). When extracellular adhesion molecules are made synthetically they are known as substrates (Lochter et

al., 1995, Kuschel et al., 2006). These substrata molecules have been extensively studied in mammalian and leech neurons, however, only a few studies have investigated their influence on adult zebrafish neurons (Nitkin et al., 1983, Fromherz et al., 1991). The roles of neuron guidance are accomplished by a number of signal pathways from extracellular space. These signals are often recognized as negative (inhibitory) or positive (promoting outgrowth of neurite) (Letourneau et al., 1994, Aplin et al., 1998, Dontchev and Letourneau, 2003).

The ECM is a mixture of proteins and polysaccharides that occupy the interstitial space in the nervous tissue (Theodosis et al., 1997, Zagris, 2001). ECM plays an essential role in providing the physical microenvironment which cells require for their optimal existence and regulates cell function (Agnati et al., 1995, Ahmed et al., 2005). Furthermore, these molecules have the ability to regulate cell behavior during both development and maturation (Sheppard et al., 1991, Watt, 2002). Neurons mature from progenitor cells, hence, the growth and development of neurons is facilitated by the ECM which target these progenitor cells and direct migration and differentiation as well as the maturation of neurons and the extension of the axons. It gives anchorage to the maturing neurites, guiding the intercellular communication (Albelda and Buck, 1990, Letourneau et al., 1994, Stockton and Jacobson, 2001).

Besides ECM proteins, surface properties such as the electrostaticity have been suggested to have effects on cell adhesion, differentiation, and growth (Springer, 1990, Adams and Watt, 1993, Schwartz and Ingber, 1994, De-Carvalho et al., 1999, Koh et al., 2008). Vital poly-electrolytes are used often in cell adhesion studies including poly-lysine. It is a cation synthetic polymer molecule which attaches to cell surfaces to mediate cell adherence and promote neurite outgrowth by electrostatic

attraction between the positively charged coverslip surface and the negatively charged cells (Yavin and Yavin, 1974, Humphries, 1996, Loftus and Liddington, 1997, Tang et al., 2007).

During development, or after injury to the nervous system, the ECM plays an important role in stabilizing the neurons and neurites branches (Reichardt, 1991, TessierLavigne and Goodman, 1996). In the cell culture system, the attachment, growth and differentiation of cells are mainly dependent upon a suitable ECM environment and polyelectrolyte polymers. Substrates are sometimes artificially made synthetic polymers rather than natural substances, thus, they do not interfere with the cell attachment process (Kleinfeld et al., 1988). Even though many studies have shown that these molecules are often used as a biomaterial surface for many cell cultures such as rat DRG or chicken neurons, there is limited evidence about their effect on the adhesion and growth of adult zebrafish neurons (Tomaselli et al., 1986, Sadaghiani et al., 1994, Agius and Cochard, 1998). This present study was designed to investigate the effects of ECM proteins such as laminin, fibronectin, collagen and polymers poly-(L-D)-lysine on the induction of neurite growth from adult zebrafish CNS neurons.

5.2 METHOD

5.2a) Dissociated adult zebrafish neuron culture

As previously described in Chapter 3, zebrafish brain neuronal cells were cultivated and grown at 28.5C with 1.5% CO_2. All the cells were grown in 4 well-plates on coverslips coated with a substrate under sterile condition for fourteen days. The coverslips were sterilised with 100% ethanol and UV light overnight and pre-wetted with sterile phosphate-buffered saline (PBS) prior to use.

5.2b) Examining the cell growth on different types of coverslips

Plastic and glass coverslips and the 4 plastic well-plates (NUNC) were pre-treated with poly-lysine (L-form) for one hour. The substrates were removed gently and the coated coverslips were air dried for 20 minutes under sterile conditions.

5.2c) Preparation of coated coverslips with different substrates

Coverslips were coated with different substrates to examine the optimum growth of neurites. Plastic coverslips (13mm diameter NUNC) were autoclaved in the dry beaker for 20 minutes. Each coverslip was then transferred to 4 well-plates prior to coating with different substrates.

5.2d) Assays for cell adherence on coverslip using different substrates

To examine the cell adhesion during axon regeneration, sterile plastic coverslips were pre-coated with different substrate conditions at 28°C. The coverslips (NUNC 13mm) were pre-coated with different substrates (Table 5.1). The solutions were incubated for three hours then aspirated. Cell suspensions of adult zebrafish neurons

were immediately plated onto these coverslips. Control coverslips were used without any adhesive molecules on their surface for comparison.

Table 5.1: The table shows the six substrates that were used in this study.

Substrates	Concentration used	Manufactures
Poly- L- lysine	100µg/mL	Sigma
Poly-D- lysine	100µg/ml	Sigma
Collagen	50µg/ml	Stem Cell Technologies
Fibronectin + gelatin	50µg/ml+100mg of gelatine in 100ml water	Sigma
Laminin	10µg/ml	Sigma
Poly-L-lysine + laminin	100µg/ml + 10µg/ml	Sigma

5.2e) Neurite Outgrowth Determination

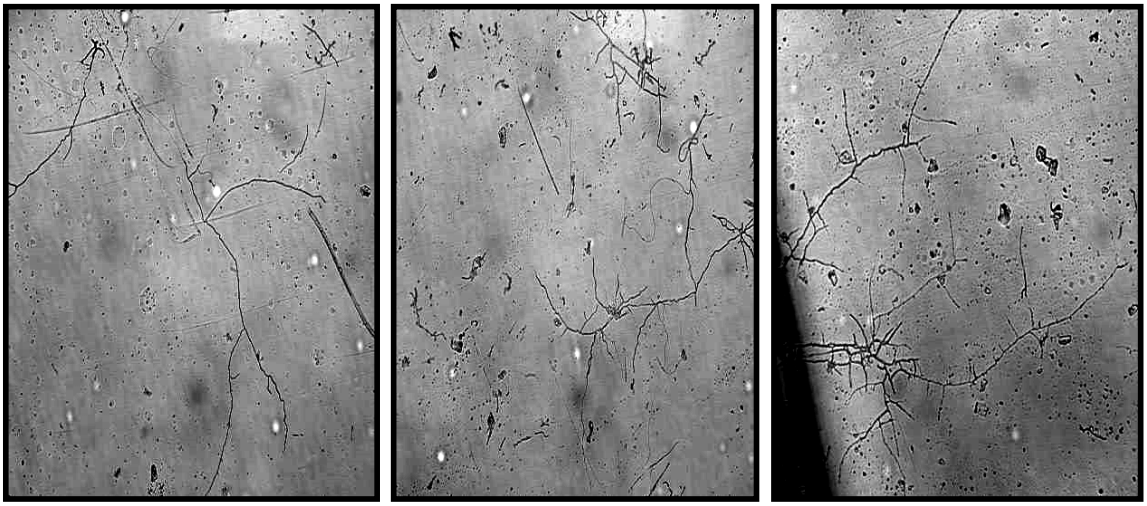
The extensions of the neurites were quantified using 1cm test grid analysis as described in detail in chapter 2. Growth was measured by counting the intersection per unit test length as described earlier (chapter 2.6). The study was repeated six times for each substrate used.

5.3 RESULTS

EVALUATION ON ATTACHMENT OF CELLS TO SUBSTRATES

5.3a) Attachment of adult zebrafish neuronal cells in different surface conditions

From the quantitative measure by grid test analysis, it was observed that adult zebrafish neurons grew and extended their neurites in all three conditions (Figure 5.2) with plastic being the most effective surface in growing this type of neuron. The extensive neurite growth was observed significantly in conditions where cells were cultured on the floor of the 4-well plates without any cover slips in comparison to wells that contained coated poly-L-lysine coverslips. The neurite growth was observed to be considerable, about 5.7±0.79 intersections per unit length of test line. Substantial neurite growth was observed for the poly-L-lysine coated plastic coverslips compared to the poly-L-lysine coated glass covers-slips. The number of intersections of unit of test line for such neurons was about 4.5±0.64 whilst neurons grown on substrate coated glass coverslips was about 3.0±0.59. The difference was statistically significant.



PLL glass coverslip **PLL plastic coverslip** **PLL on well-plate surface**

Figure 5.1: The effect on neurite growth of adult zebrafish on plastic and glass environment.

These examples of micrographs showed that zebrafish neurons were capable of growing neurites on both glass and plastic surfaces. However, the extent of neurite growth was greater for those neurons grown on the poly-L-lysine coated floor of the plastic 4 well-plates. Quantitative analysis showed that the number of intersections of unit of test line for such neurons was about 5.7±0.79. This compared to about 3.0±0.59 for neurons grown on poly-L-lysine coated glass coverslips.

5.3b) Attachment of cells to substrates

Figure 5.3 illustrated the growth of neurites using different substrates and combination of substrates. The results indicated that the combination of fibronectin-gelatin coated coverslip produced the greatest amount of neurite growth for the adult zebrafish CNS neurons. The qualitative analysis measured shown that the number of intersections per unit test line was about of 6.2±0.91compared to the control of about 3.8±0.41. The differences were statistically significant.

Similarly, a direct observation of adult zebrafish culture on mixture of poly-L-lysine and laminin coated substrate produced fairly extensive neurite growth. The number of intersections of unit of test line for such neurons in this two substrate conditions were about 5.7±0.44 compared to the control of about 3.8±0.41. Poly-L-lysine alone and laminin alone also resulted in a moderate degree of enhancement of neurite growth intersections about 4.9±0.66 and 4.8±0.69, respectively. The differences were statistically significant. Both collagen and poly-D-lysine produced no significant increase in neurite extension to that seen on untreated coverslips, with the intersection approximately about 3.9±0.27 and 4.0±0.43, respectively, compared to the control of 3.8±0.41. (P-values are described in Appendix 8)

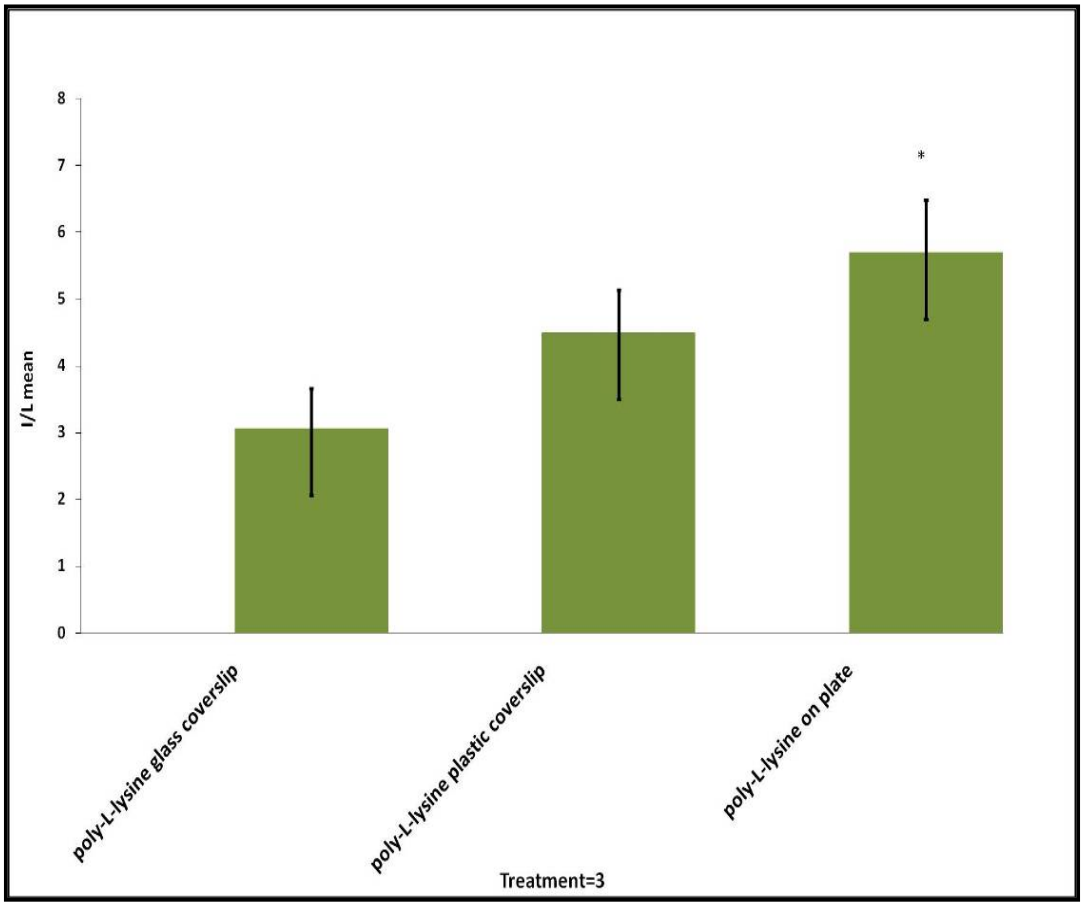

8
7
6
5
4
3
2
1
0
I/L mean
*
poly-L-lysine glass coversilp
poly-L-lysine plastic coversilp
poly-L-lysine on plate
Treatment=3

Figure 5.2: The effect on neurite growth of adult zebrafish on different surface conditions.

The graph illustrated the extent of neurite growth in the presence of the different conditions. Each bar represents the mean ± SEM of three preparations. The well-plate coated with poly-L-lysine was shown to give the best growth compared to the coated coverslips. Neurite growth was reduced when glass coverslips were used as a substrate.

*$p<0.05$

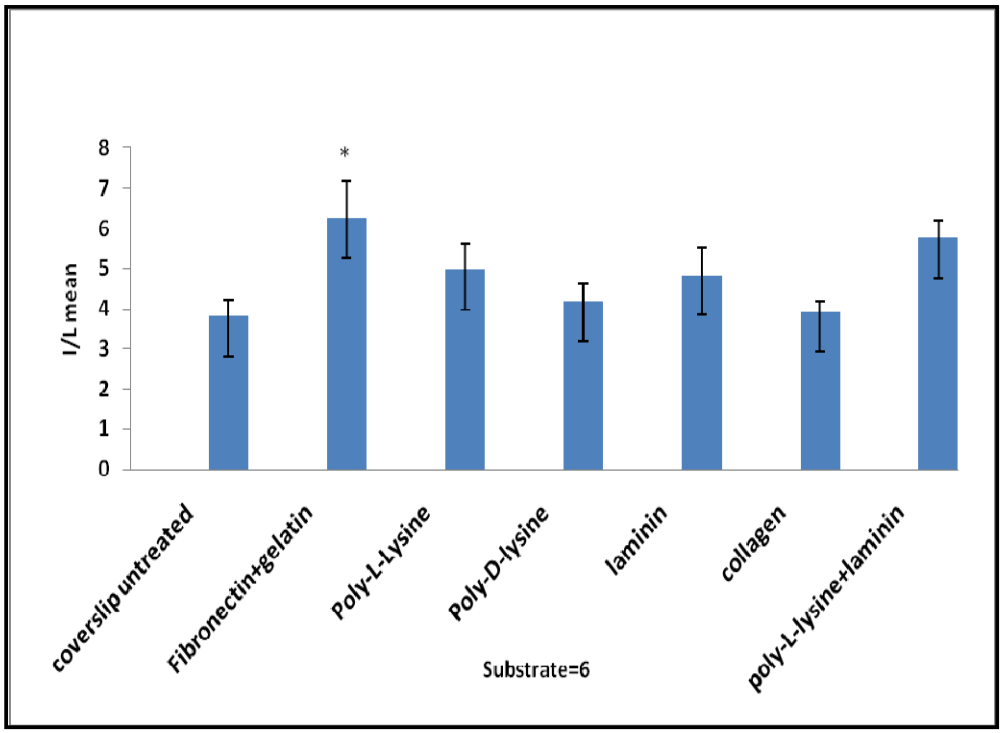

8
7
6
5
4
3
2
1
0
I/L mean
*
coverslip untreated
Fibronectin+gelatin
Poly-L-Lysine
Poly-D-lysine
laminin
collagen
poly-L-lysine+laminin
Substrate=6

Figure 5.3: The effect of different substrates on adult zebrafish neurite growth.

The graph is a representation of the extent of neurite growth using different substrates in comparison to the controls. It is apparent from the graph that fibronectic-gelatin stimulated the highest growth of neurites while Poly-D-lysine and collagen produced the lowest growth rate. Each bar represents the mean ± SEM of six preparations. (P-values are presented in appendix 8).

*$p<0.05$ compared to the untreated group

5.4 DISCUSSION

This study has demonstrated that the optimal condition for neurite growth from zebrafish CNS neurons was to culture without coverslips. The surfaces of pre-coated 4-well plates were found to be the most suitable condition in this comparison. This study has also shown that there are differences in neurite outgrowth in untreated plastic well-plate, plastic and glass coverslips pre-treated with the substrate. However, choosing to culture adult zebrafish neurons without coverslips would create difficulties at later stages as the experiments would require that the neurons and their regenerated neurites be labelled subsequently using immunohistochemical techniques, hence, it is necessary to grow the neurons on some type of coverslip for ease of microscope handling. The second part of this study clearly established that a substrate consisting of a mixture of fibronectin and gelatin produced the greatest amount of neurite regeneration from adult zebrafish CNS neurons.

The findings of the second part of this study illustrated that plastic coverslips coated with a mixture of fibronectin and gelatin may be the best potential substrate to use in the future studies of adult zebrafish CNS neurons. Poly-L-lysine was used as the substrate in my previous study (study 1 and study 2) to pre-coat the coverslips for adult zebrafish neuronal cells.

Poly-L-lysine caused the adult zebrafish neuronal cells to adhere to the surface of the coverslips, thereby, promoting the outgrowth level of neurites moderately compared to fibronectin-gelatin substrate. It is believed that the axonal regeneration from both PNS and CNS neurons take place upon the cell surfaces or within ECM which helps in maintenance and growth of the cells (Carbonetto et al., 1987, Burden-

Gulley et al., 1995). ECM is comprised mainly of glycoproteins, collagen, elastin, microfibrillar protein, proteoglycans including hyaluronan that are well accepted to be a family of neurite-promoting agents (Manthorpe et al., 1983).

The attachment of cells to a substrate is a requirement for the survival and proliferation of most cell types (Frei et al., 1992). It has been shown that there are three main phases of cellular events that take place in cell adhesion and these are described in the diagram below (Figure 5.4).

PHASE 3

Formation of specialised contact between cell and substrate surface

PHASE 1

Cell attaches to the substrate

PHASE 2

Cell spreads and grows on the substrate

Figure 5.4: General cellular processes of cell adhesion to the substrate-bound coverslip.

The flow chart diagram (Figure 5.4) describes the adherence of cells on the pre-coated substrate coverslips. Initially, the round cells attach to the substrate that has been coated on the coverslip. This process causes the binding interaction between cell surface receptors and the ligands present on the substrate (Burridge et al., 1988, Woods and Couchman, 1988, Dertinger et al., 2002). In the second phase, cells may start to proliferate or extend their growth on the substrate-coated coverslips (Grinnell, 1978, Aplin and Hughes, 1981). However, it is still unclear whether the spreading of the cells occurred due to the substratum-bound ligands interacting with the surface receptors and intracellular cytoskeleton or whether the ligands have the ability to cause cell spreading. This mechanism has not yet been fully explained (Gullberg and Ekblom, 1995, Stockton and Jacobson, 2001).

The third phase of this process involves the development and distribution of specialized contacts between the cell and the adhesive substrate leading to interactions between intracellular cytoskeleton and the extracellular molecules. The neurons were then guided to the correct target by responding to the variety of guidance molecules and triggered the growth response. Further research is required in order to define the exact mechanism of this cellular interaction process (Couchman and Rees, 1979, Couchman et al., 1982).

The purpose of this study was to determine whether substrates other than poly-l-lysine may have produced a greater effect in supporting or enhancing neurite regeneration for these adult zebrafish CNS neurons. The study was optimized in several pilot experiments and noted that poly-L-lysine did not result in a full adherence of all cells to the substrate. Many cells appeared to detach easily and were subsequently lost as they were unable to extend their neurites. Six adhesive substrates including laminin, poly-L-lysine, poly-D-lysine, fibronectin, with gelatin as well as laminin combined with poly-L-lysine were systematically examined. As indicated earlier, fibronectin and gelatin coated coverslips caused the greatest degree of neurite regeneration in this case. Fibronectin is a prominent component of ECM, cell surface and plasma. Frost and Westerfield (1986) has reported that there was an outstanding correlation between the distribution of adhesive substrate molecules and the outgrowth of motor growth cones from zebrafish embryos (Frost and Westerfield, 1986). It was observed that embryonic motor neuron growth cones favoured interaction with laminin, but not fibronectin, during the outgrowth process (Frost and Westerfield, 1986, Rogers et al., 1986, Westerfield, 1987). This is in contrast to the present study where fibronectin in combination with gelatin produced the greatest degree of neurite outgrowth from adult zebrafish CNS neurons. This may show either the differences between various types of zebrafish neurones or may demonstrate differences due to the age of the zebrafish used in the two studies. A possible explanation why fibronectin-gelatin had the strongest effect in this regard may be because of the composition of gelatin or the fact that the CNS tissue of adult zebrafish contains different compositions of ECM and may prefer different cell adhesion substrates.

The fibronectin molecule is classified into dimeric glycoprotein group containing different sites. The C-terminus of fibronectin is responsible for binding interactions with other integrins and proteoglycans, while the N terminus contain the domains which regulate the binding of collagen and gelatin (Akimov and Belkin, 2001, Peters et al., 2003, Bingham et al., 2008, Kadler et al., 2008). It has been reported that fibronectin plays a vital role in influencing cell growth and prevents apoptosis by causing interactions of cell-binding with integrins (Zigmond, 1996, Frisch and Ruoslahti, 1997). The cross link of fibronectin and gelatin combined might possibly facilitate cell attachment activity of adult zebrafish neurons by increasing the binding affinity on the surface to the growing cells (Carter et al., 1981).

Laminin is one of the most important adhesion molecules that is currently being investigated. It serves a vital role in both developing and mature neurons as well as regulating axon growth and differentiation (Kleinman et al., 1985, Martin and Timpl, 1987). In this present study, Laminin was also found to induce a reasonable degree of neurite extension from both mammalian and adult zebrafish neurons. It has previously been shown to have a similar effect on embryonic and developing zebrafish neurons (Paulus and Halloran, 2006).

Another interesting finding by (Campbell and Chader, 1988, Cormie and Robinson, 2007) demonstrated that embryonic zebrafish neurons grow effectively on laminin coated glass coverslips. Other coatings such as fibronectin-coated glass coverslips or uncoated plastic and glass coverslips showed lower number of neurite outgrowth (Cormie and Robinson, 2007). Recently, it was shown that different range of acidity prepared for laminin used for coating coverslip might have some control on neural outgrowth. It was found that when inducing the pH level to acidity on laminin surface

coating coverslip, some neurons were attached to the surface extensively, resulting in greater outgrowth compared to the neural coating surface (Roach et al., 2010).

Fibronectin, laminin and collagen belong to the ECM family and serve critical functions in cell attachment, growth, migration as well as differentiation of many cell types (Menko and Boettiger, 1987, Bidanset et al., 1992, Garcia et al., 1999). Poly-lysine (L and D) are synthetic polymer used to facilitate and accelerate cell attachment to both glass and plastic surface. The cationic poly-electrolytes Poly-L and D-lysine have the same roles as ECM-type substrates which are to promote cell adhesion by the electrostatic attraction between the positively charged biomaterial surface and the negatively charged cells. In addition, poly-lysine also helps the absorption of serum or extracellular matrix protein to culture substrate. In the coating process of coverslip, both type of poly-lysine are widely selected in coverslip coating process. The main difference between this poly-D lysine and poly-L-lysine is the sterical structure of atoms in the molecules. Poly-D lysine is not broken down or attacked by proteases while poly-L-lysine degrades by cellular proteases. It can be seen that adult zebrafish neurons favour poly-L-lysine over poly-D lysine in regards to the level of subsequent neurite extension achieved (Manthorpe et al., 1983, James et al., 2000). In this study, it was found that both poly-L and D-lysine only had a moderate effect in promoting neurite outgrowth. A possible explanation may be that many neuronal cells appeared to detach themselves from the surface of the coverslips with relative ease over the first few days of incubation and were, therefore, unable to regrow their neurites. Appropriate poly-lysine concentrations are necessary for proper neurite outgrowth since low concentrations do not properly promote cell adhesion and high concentrations often result in cell lysis. An

intermediate concentration is, therefore, preferred as cells can spread uniformly (Hategan et al., 2004).

In addition, there was no significant effect on neurite extension when laminin and poly-L-lysine were combined in comparison to those coated with cation polymers substrates or laminin alone. This suggests that combining the ECM protein with cationic polymers reduces the charges of polyelectrolytes and possibly causes the lower affinity in binding of cell surface receptors to the laminin substrate (Chang and Sretavan, 2008). Moreover, it explains why the combination of these two substrates only produced a moderate effect on neurite extension. Collagen alone, on the other hand, had no significant effect on neurite growth in comparison to the control condition.

Collagens are an extracellular protein with a triple helix structure. There are 28 collagen types which differ slightly in size, structure and function (Kadler et al., 1996, Hubert et al., 2009). Even though collagen is known to be quite an effective adhesive substrate for many types of neurons, my present results indicated that it is not the best substrate for adult zebrafish neurons. Collagens are a major component of ECM in most tissues but are rarely present within the substance of the mature CNS. It is, however, found in some of the meningeal membranes surrounding the brain and spinal cord and is associated with peripheral ganglia and nerves (Weller, 2005). Therefore, most CNS neurons may not possess the requisite receptors that can act as ligands for collagen. The role of the substrate in the tissue culture of neuronal cells is crucial for their proper development, maturation and growth (Chang and Sretavan, 2008, Yoo and Nam, 2012). However, each neuronal cell type has its favoured substrates to which it best responds for optimal adhesion and neurite

extensions (Carbonetto et al., 1983, Hynes, 1992). There are two major requirements for neurite to grow successfully both *in vivo* and *in vitro*. These are trophic factors that enhance the survival of neurons as described in chapter 4 and the substances that act as a supporter for neurite enhancing factors (Varon and Bunge, 1978). These supporting agents could be ECM constituents such as fibronectin, gelatin and Laminin (Reichardt, 1991, Whitworth et al., 1995, Patist et al., 2004). However, for most neuronal cell types, poly-cationic adhesive substrates are commonly used as they provide rapid cell attachment in tissue culture (Letourneau, 1975). Adherence of neurons to the tissue culture plate is critical for the successful regeneration of neurites as increased adherence generally leads to enhanced neurite regeneration (Kaech and Banker, 2006) .

Remarks should be made concerning the concentration of the various adhesive substrates used in tissue culture. Those used in the present experiments were based on a survey of the literature in the field. However, there appears to be a lack of systematic study on the optimal concentrations and appropriate types of substrates that should be used for particular type of tissue cultures including for adult zebrafish CNS neurons. This is an area ripe for further investigation.

Different neuronal types might have different sets of surface receptors for various adhesive substrates (Buck and Horwitz, 1987, Albelda and Buck, 1990). The experiments in this report revealed that whilst the different substrates examined produced a different amount of neurite extension, they did not alter the morphology of the neurites or their extensions in any obvious way. The neurons grew and extended their neuritic network from the cell bodies in many directions. This contrasted with the study by (Payne et al., 1992). It was postulated that different

adhesion molecules had different effects on growth cone morphology in response to modulate the substrate composition. Previous work suggested that neuronal cell cultures obtained from amphibians (Xenopus laevis) grew sufficiently on plastic coverslips coated with substrates such as laminin and poly-L-lysine (Cormie and Robinson, 2007). This is similar to the observations made in the present study that a plastic substrate is the most favourable surface for growing adult zebrafish neuronal cells.

In conclusion, Adult zebrafish neurons were attached mostly to the surface of coverslips coated with fibronectin and gelatin compared to other substrates used after seven days. Despite the presence of dissociated neurons (detached and floating) on the plate and in the media, significant growth of adult zebrafish neuronal cells were observed. It was observed that fibronecin and gelatin enhanced cell attachment on the coverslip, causing an increase in neuronal growth.These experiments have shown that substrate molecules are essential in promoting the adult zebrafish neuronal growth; therefore, fibronectin-gelatin substrate should be used in future experiments.

CHAPTER SIX

GROWTH OF DISSOCIATED ADULT ZEBRAFISH CNS NEURONS ON RAT CNS TISSUE CRYO-SECTIONS

6.1 INTRODUCTION

It has long been known that neuronal axons typically fail to regenerate after lesion within the mature mammalian CNS. Many factors are thought to be responsible for the failure of axonal regeneration. Research has conclusively shown that this failure of axon regrowth and elongation is not due to the intrinsic properties of the neurons (Liu and Chambers, 1958, Richardson et al., 1980, Fournier and Strittmatter, 2001, Taylor et al., 2006). Evidence suggests that the environment provided by the mature mammalian CNS is not conducive to support such axonal regeneration. However, the factors which influence the exact nature of the CNS environment which limits such regeneration are yet to be determined (Horner and Gage, 2000, Liu et al., 2008, Sun and He, 2010). It is thought that to achieve successful axonal regeneration in the mammalian CNS, the neurons have to be provided with appropriate growth factors (e.g. neurotrophic factors) whilst at the same time, local inhibitory factors that may be present in the post-lesion CNS environment are removed or neutralised in some way (Schnell and Schwab, 1990, Kawaja and Gage, 1991, Bradbury et al., 1999, Coumans et al., 2001, Schwab, 2002, Sandvig et al., 2004). Injury to the CNS causes a sequence of cellular changes to occur including the inflammatory response within the proximity of the injury. In addition, injury causes the breakdown of the myelin from the injured axons and this is thought to release various oligodendrocyte-related factors that are inhibitory to subsequent axonal regeneration (Faulkner et al., 2004). Part of the response to the CNS injury also involves the formation of glial scar tissue as a result of astrocyte activity (Windle and Chambers, 1950, Davies et al., 1999, Faulkner et al., 2004). This glial scar tissue is thought to prove a physical and probably, chemical barrier to successful axonal regeneration (Busch and Silver, 2007). Astrocytes have been shown to cause

contact-mediated collapse of axonal growth cones under certain conditions (Kerschensteiner et al., 2005). Therefore, current research in this field is directed towards altering the post-injury CNS environment to make it more conducive in supporting axonal regeneration (Fernandes et al., 1999, Bulsara et al., 2002, Bunge, 2008, Kim et al., 2012).

In contrast to mammals, zebrafish are able to regrow their severed axons in the CNS including their visual pathway spontaneously, leading to the recovery of function. The CNS environment in zebrafish is growth permissive possibly because of the relative lack of the same inhibitory molecules known to be present in the mammalian CNS (Becker et al., 1997, Veldman et al., 2007). It is believed that there are substantial numbers of myelin and myelin based proteins in both lower vertebrates and in mammals. Interestingly, there appear to be few molecules such as unglycosylated hydrophobic proteolipid proteins that are absent or inexpressive in the zebrafish CNS, but are present in the mammalian CNS (Waehneldt and Jeserich, 1984). It may be that these molecules are instrumental in creating the non-permissive environment in the mammalian CNS by acting as inhibitors to the regrowth of axons (Becker and Becker, 2002, Schweitzer et al., 2006).

The question arises whether or not the environment provided by the mammalian CNS can support axonal regeneration from adult zebrafish neurons. It has previously been shown, using a cryoculture technique, that rat CNS tissues cannot support axonal regeneration from adult mammalian CNS neurons (David et al., 1990, Bedi et al., 1992, Yiu and He, 2006, Liu et al., 2008). However, it is possible that zebrafish CNS neurons do not have the receptors present on their surface that would react to the inhibitory factors thought to be present in the mammalian CNS tissues. If so, it could be predicted that adult zebrafish CNS neurons should be able to extend their

neurites on mammalian CNS tissue sections. The study reported in this chapter was designed to examine this possibility.

6.2 METHODS

6.2a) Cryosection of substrate tissue

Cryostat sections of various brain regions were rapidly frozen (as described in Chapter 2.5-2.6). Prior to culture, these sections were placed on sterilised Poly-L-lysine coated coverslips as the substrate onto which dissociated adult zebrafish CNS neuronal suspensions were plated. These cultures were incubated at 28.5^{o}C in an atmosphere containing 1.5% CO_2 for fourteen days. The cultures were then fixed in 2% paraformaldehyde for 20 minutes followed by fixation in ice-cold methanol, again as described in detail in Chapter 2

6.2b) Staining the sections with Toluidine blue

In some instances, cryostat sections of rat brain tissues which had been picked up on glass coverslips were stained with warm 1% toluidine blue for two minutes to examine their general morphology. Such sections were subsequently washed briefly in running tap water before being mounted onto glass slides and viewed under a standard transmission light microscope (Vickers M17).

6.2c) Immunolabeling the sections with antibodies

The sections and overlying zebrafish CNS neurons were immunolabeled with double labelling procedures. Rat CNS sections were labelled with anti-mouse GFAP and the regenerating zebrafish neurons were labelled with GAP43 antibody (see table 6.1). Subsequently, the detection of primary antibodies was performed by conjugating secondary antibodies that are specific for certain primary antibodies. Binding, expression and cell growth were visualised using different flurophores dyes (red,

green). These fluorescent, immune-labeled sections and cultures were viewed under an Olympus fluorescent microscope using the appropriate filters. The exact protocol and antibodies used are given in Appendix 5 and 6

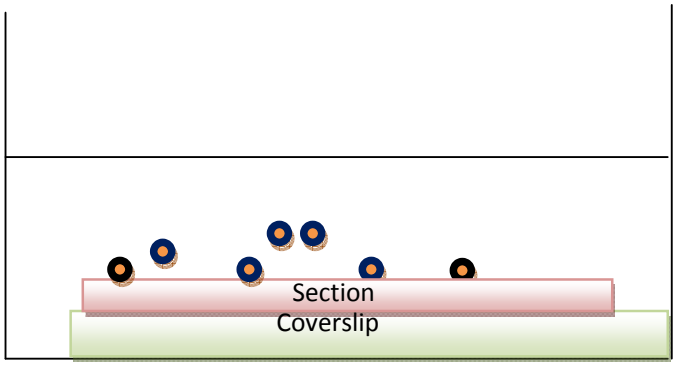
Table 6.1: Primary and Secondary antibodies used for staining.

Primary Antibodies	Secondary Antibodies
• **Primary antibody rabbit polyclonal anti GAP-43 (abcam)** • **Primary Mouse monoclonal GFAP (Sigma)**	• **Goat anti rabbit IgG conjugated biotin (abcam)** • **Goat anti mouse IgG conjugated with cy (Sigma)**

A considerable amount of time was spent in determining the suitable primary antibody that was most appropriate in expressing zebrafish neurite outgrowth. For many months, rabbit polyclonal GAP-43 antibody (G9263; GAP-7B10 Sigma St. Louis, MO) was used to label the outgrowth. However, this antibody was found to give inconsistent results and often failed to label zebrafish growth associated protein although it could be used to label such rodent proteins. This led to the time-consuming examination of a number of commercial anti-GAP43 antibodies at various dilutions. It was determined that the antibody that provided the most successful immunolabeling for zebrafish growth associated protein was the specific rabbit polyclonal GAP-43 (Anti GAP43 antibody (ab75057 Cambridge, MA; Abcam). This antibody was found to react specifically with zebrafish growth associated protein.

Anti-GAP-43 antibodies purchased from other suppliers were often found to not react with zebrafish growth associated protein although they could often be used to label such rodent proteins.

A



B

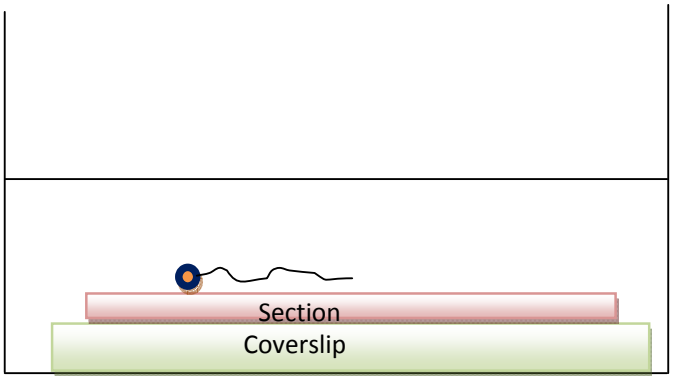


Figure 6.1: diagram to illustrate the cryo-culture techniques.

This diagram shows a representation of a coverslip with a cryostat section mounted onto its surface. The coverslip has been placed in a tissue culture well and a suspension of dissociated zebrafish CNS neurons has been introduced into the well. (Figure 6.1a). After a short period of incubation, some of the disassociated cells come to settle over the cryostat section and may or may not begin to extend neurites. The figure 6.1b depicts such a neuron which has begun to extend a single neurite.

6.3 RESULTS

6.3a) Staining of Rat Brain Sections

Ten micrometre-thick sections through frozen rat cerebellum were stained with toluidine blue and examined under a Vickers M17 transmission light bright field microscope. Sections which were of poor quality or which contained artefacts produced due to the freezing procedure resulted in those blocks of tissue to be discarded. Good quality blocks were serially sectioned and such sections were picked up on poly-L-lysine coated glass coverslips and used as substrates onto which zebrafish CNS neurons were plated. Figure 6.2 shows a frozen section of the cerebellum that has been stained with toluidine blue. It was observed that the areas of grey and white matter were relatively difficult to distinguish in such toluidine blue-stained sections.

Micrographs in figure 6.3-6.4 show the immunolabeled images from cerebellum and cortex of rats. The sections were all viewed under and Olympus fluorescent microscope to examine the general distribution of GFAP immunolabeling of rat brain sections. Figure 6.5 showed micrographs of the growing adult zebrafish neurons labelled with GAP-43. The staining clearly illustrated that adult zebrafish neurons were capable of regenerating the new neurites extensively on the 4 well-plates. The neurites extended from cell bodies and branched out their network on the rat brain cryosection. The growth displayed was quite extensive in all directions, and some interconnectivity between the neurons appeared to be present.

6.3b) Staining of adult zebrafish neurons on rat substrates

In Figure 6.6, rat cerebellum sections were attached to coated poly-lysine coverslips. The adult zebrafish CNS neurons were seen to have grown extensive neurites across the section. The regenerating neurites appeared to be quite long with several small branches. These neurites appeared to have little difficulty in growing across cryostat sections through rat brain tissues such as the cerebellum. Figure 6.7 illustrates the immunolabeled image of co-culture labelling with GFAP for the section and GAP-43 for the growing neurites. It was clearly visible that the neurites (red) were branching out from the coated poly-lysine coverslip side and were capable of growing across the sections. The results indicated that there was extensive regeneration and branching outwards on the poly-L-lysine coverslip as well as onto cryostat sections of rat CNS tissue sections.

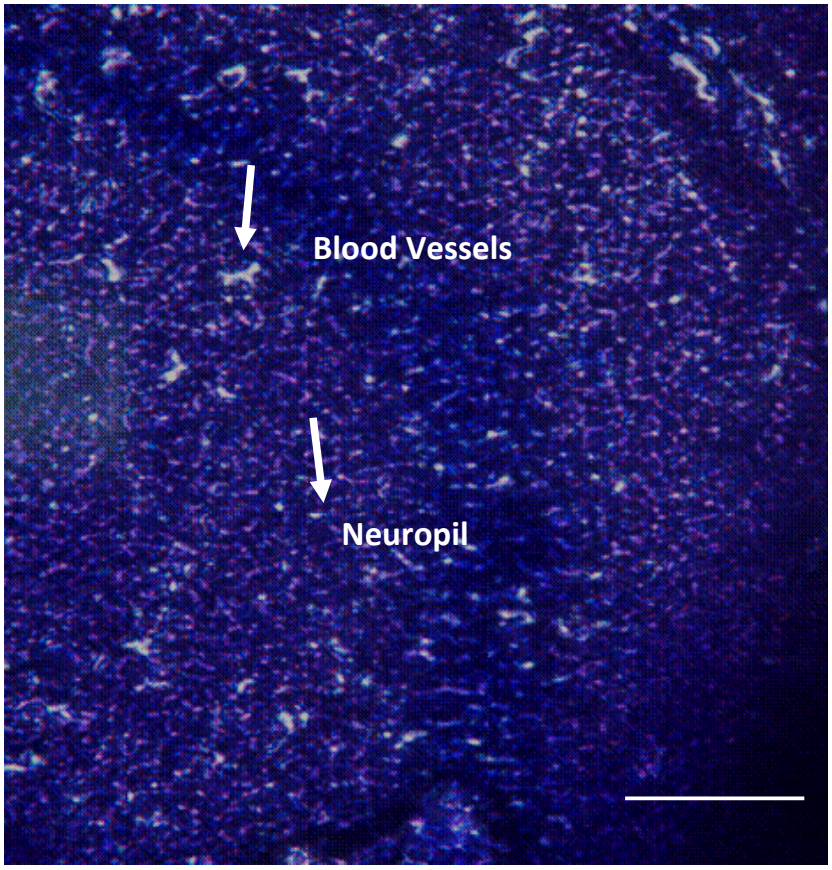
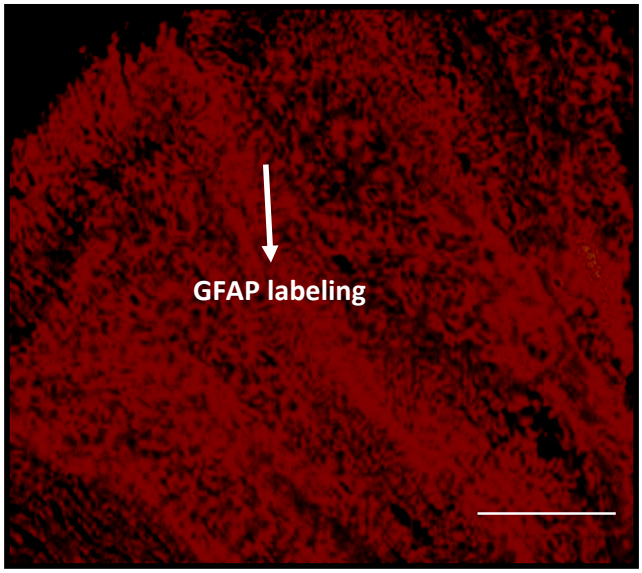

Blood Vessels
Neuropil

Figure 6.2: Toluidine blue stained transverse sections of cerebellum of adult rat.

A cerebellum was obtained from adult Wistar rat and sectioned in a cryostat at 10μm thickness. The section was stained with Toluidine Blue for few minutes before viewing with bright field microscope (scale bar = 50μm). The section shows numerous blood vessels with interspersed neuropil consisting of neurons, axons, dendrites and glial cells.

Arrows- represent blood vessels and neuropil seen on the section

A



B

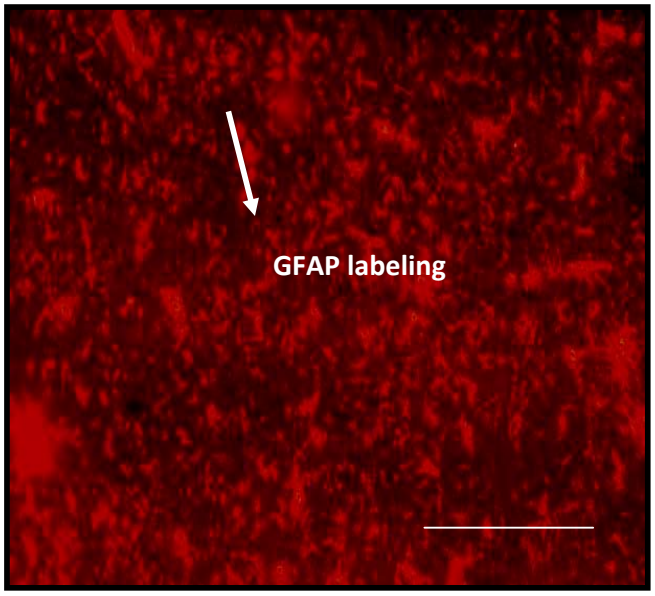


Figure 6.3: GFAP staining of cerebellum.

A cerebellum of adult Wistar Rat was sectioned out at 10μm and was stained with anti-mouse GFAP immuno-labelled, with IgG Cy 3 conjugate. Extensive areas of positive GFAP staining can be seen in micrographs a) and b).

Arrows – represent the section labeling with the antibody.

Micrograph a: Scale bar = 125μm

Micrograph b: Scale bar = 50 μm

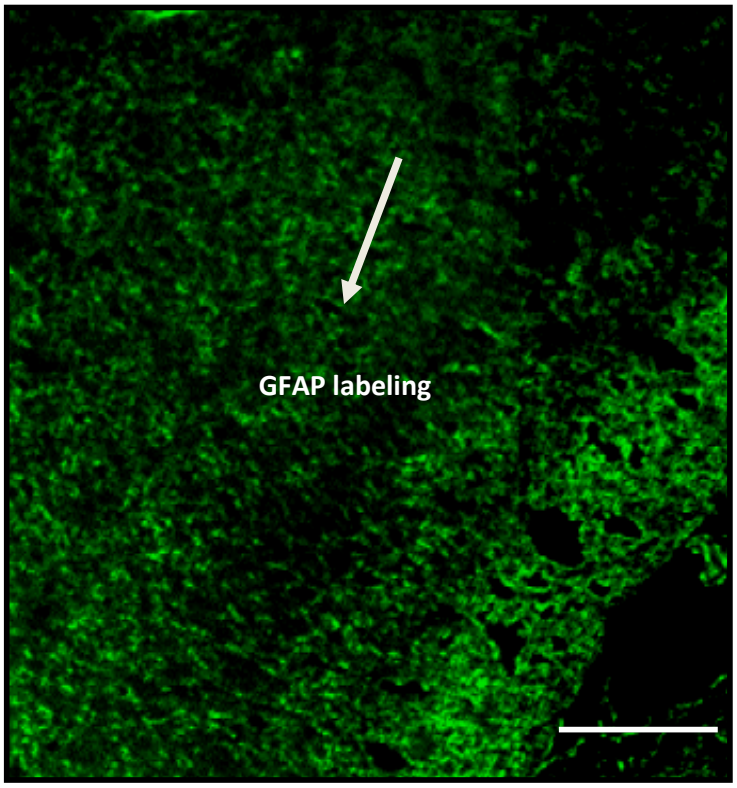
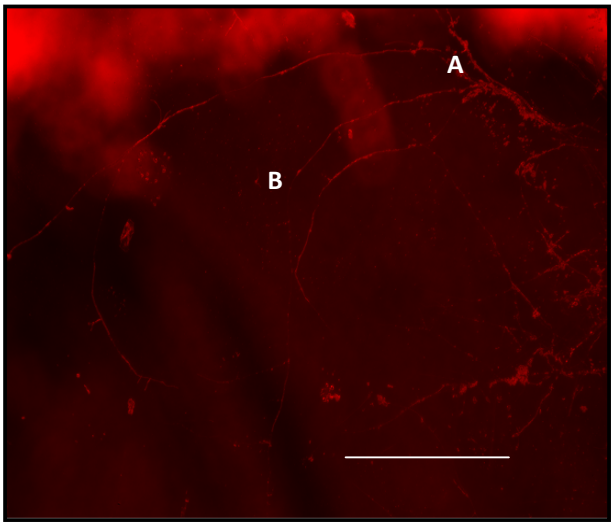

GFAP labeling

Figure 6.4: GFAP staining of rat cortex

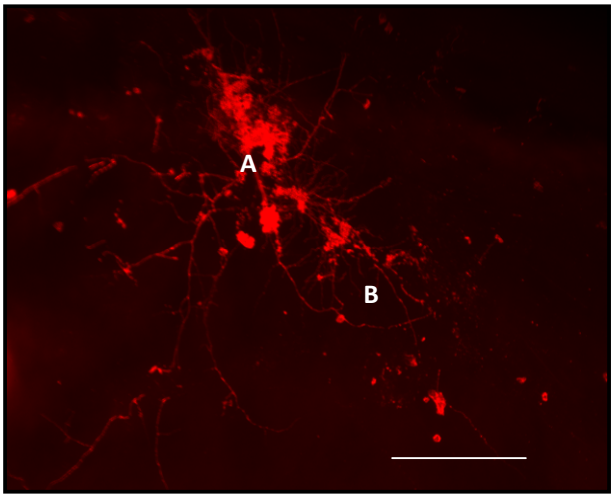
A cortex of adult Wistar rat brain was sectioned out at 10μm and stained with anti-rabbit GFAP immuno-labelled, with conjugated biotinylated FITC secondary antibody (scale bar = 100 μm). Extensive labelling can be seen throughout the section.

Arrow – represents the section labeling with the antibody.

A



B



C

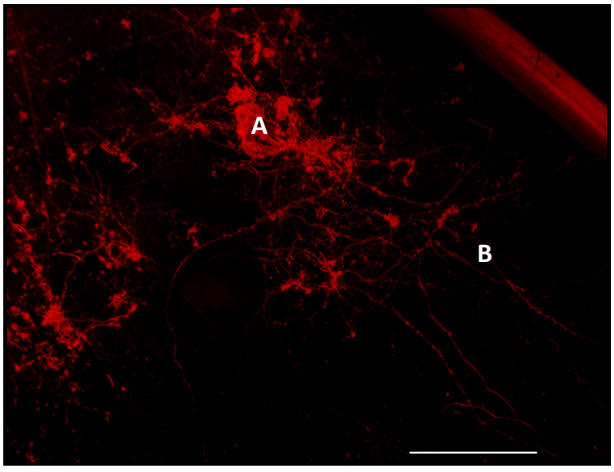
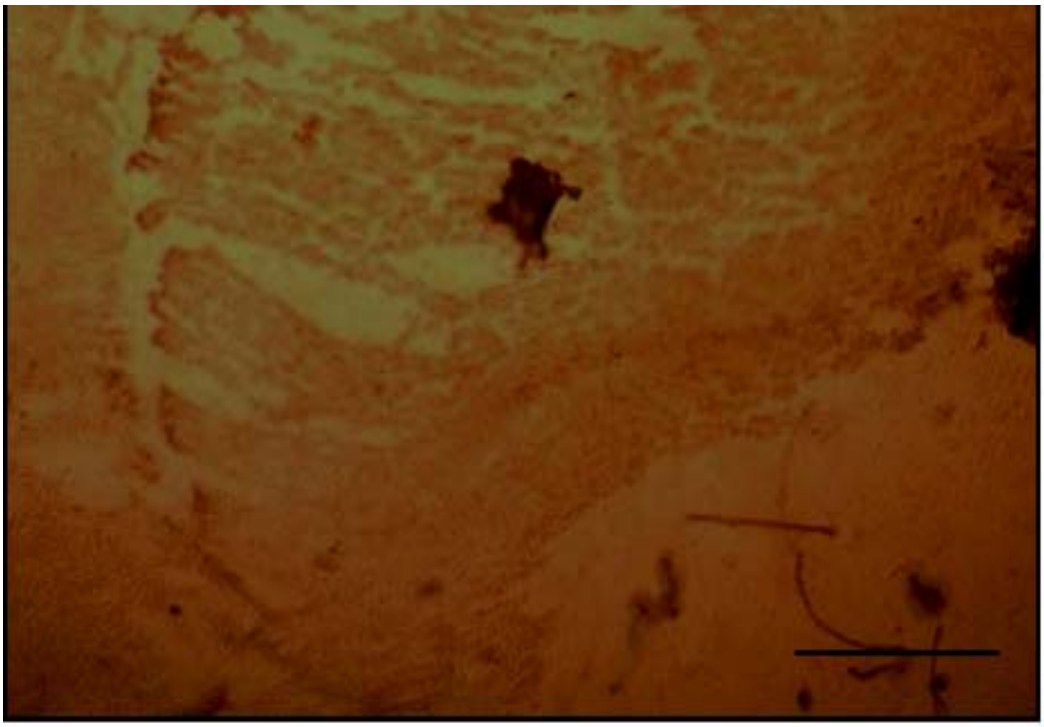


Figure 6.5: GAP-43 staining of adult zebrafish CNS neurons.

Micrograph a, b and c shows GAP-43 positive zebrafish CNS neurons and their extending neurites. Labelling was carried out after fourteen days of growth in tissue culture. A = cell bodies B = neurites (scale bar = 50µm)



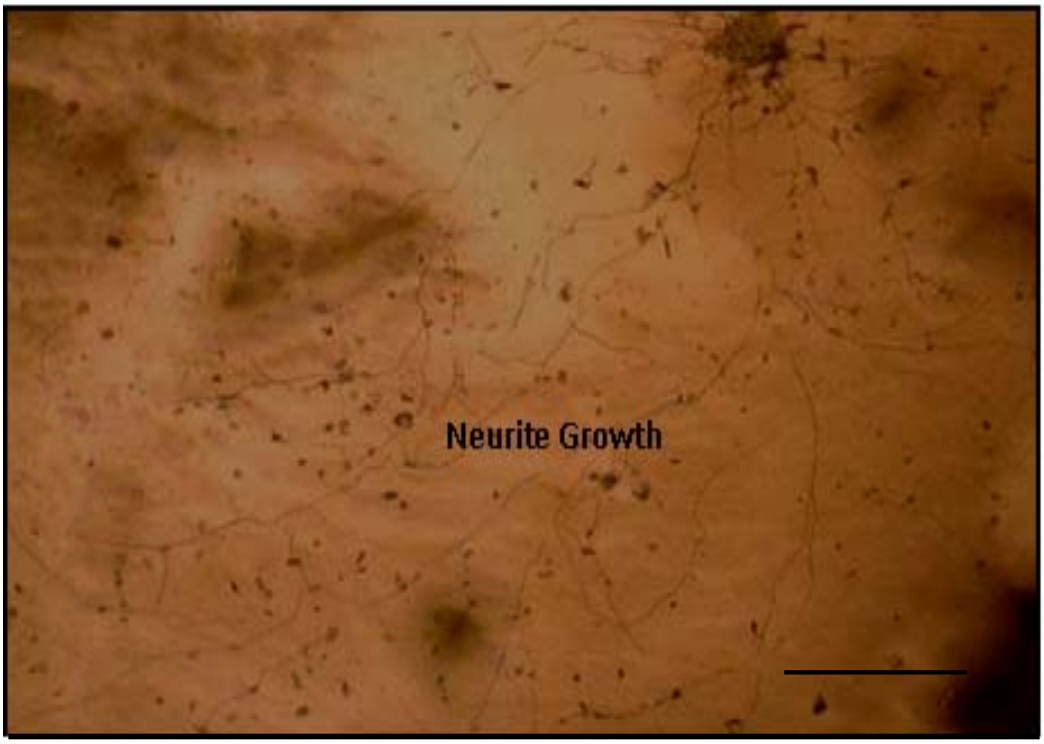

Neurite Growth

Figure 6.6: Growth of dissociated adult zebrafish CNS neurons on coverslip, with cerebellum section underneath.

a) High power micrograph of a section through the cerebellum viewed under phase contrast microscope before immune-labelling staining with antibodies. The neurites are not visible on the section. Some part of the section had been floated off with the media culturing the neurons after week of incubation. However when changing to the lower power of this section b) showed a number of adult zebrafish neurons had landed on the cerebellum section and had proceeded to grow extensive neurites on the section surface. Many neurites were also seen growing in close proximity to the section and in small gaps within the section that had arisen as artefacts during the sectioning process. (Scale bar =100μm).

A= neurites; B= section

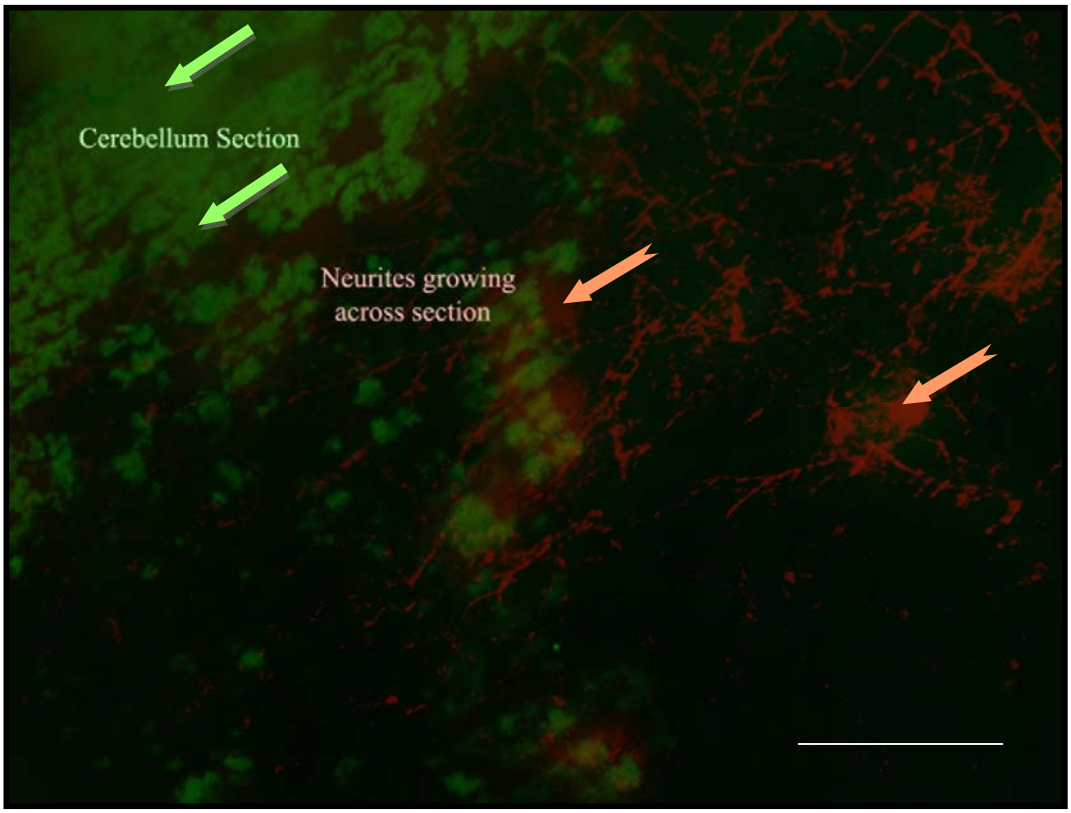

Cerebellum Section
Neurites growing
across section

Figure 6.7: Growth of dissociated adult zebrafish neurons on coverslips with a cryostat section of rat cerebellum as substrate.

Many neurites (pink arrows) were observed extending from the coverslip towards and onto the cerebellum section indicating that the section does not appear to be repulsive to the growing neurites. Neurites from adult zebrafish CNS neurons can be seen growing across the section to some extent and some appear to have branched out in several directions (green arrows). The rat cerebellar section was stained with GFAP and the zebrafish CNS neurons were counterstained with GAP-43. (scale bar = 50μm)

6.4 DISCUSSION

Convincing evidence suggests that dissociated adult zebrafish CNS neurons are capable of extending neurites on to cryostat sections taken from various regions of the rat cerebral hemispheres, cerebellum and brain stem. It has previously been shown that such tissue sections fail to support neurite extensions from disassociated rat DRG cell neurons or other types of mammalian-derived CNS neurons (Bedi et al., 1992, Shewan et al., 1993, Shewan, 1994, Shewan et al., 1995). The fact that adult zebrafish CNS neurons are capable of extending neurites on such tissue sections indicates that the environment within these sections is not as inhibitory to neurite extension from zebrafish neurons even though it may be from mammalian derived neurons. The possible reasons for this differential response need to be considered. It is possible that zebrafish neurons do not have the receptors available on their surface to react to the inhibitory molecules present in the mammalian CNS environment. In the absence of such receptors, it could be predicted that zebrafish CNS neurons would be able to extend neurites in an environment which may at the same time, be hostile to such extension from mammalian CNS neurons.

A number of studies (shown in table 6.2) have investigated cryoculture of adult CNS tissue and rat neurons to determine the functional recovery of these neurons after injury. These previous studies have provided evidence that cryosections of adult rat CNS does not support neurite regeneration from rat DRG and central neurons. The lack of such neurite regeneration in such circumstances has been generally ascribed to the presence of myelin inhibitory factors such as MAG, OMpg, Nogo and upregulation of proteogylcan (CSPG) present within the mammalian CNS environment in rats (Yiu and He, 2006). Hence, alterations to the CNS environment

after injury may influence the growth of neurites (Chapter 1 provides more detail on each inhibitory molecule that plays a role in limiting axonal regeneration).

Table 6.2: Summary of the major co-culture experiments that have previously been investigated on the effect of axonal growth inhibition.

Group	Neurons used	Substratum	Growth Response	References
Bedi	Adult Rat DRG	Adult intact rat sciatic nerve Adult lesioned rat sciatic nerve	No growth Growth	(Bedi et al., 1992)
David	Embryonic chick DRG explants	Lesioned adult rat optic nerve Adult rat optic nerve	Growth visible near lesion sites No growth	(David et al., 1990)
Shewan	Perinatal rat DRG	Adult lesioned rat sciatic nerve Adult intact sciatic nerve, perinatal and adult rat optic nerve	Growth No growth	(Shewan et al., 1993)
Crutcher	Embryonic chick sympathetic ganglion explant	White matter of adult rat brain and spinal cord Grey matter of adult rat brain and spinal cord	Poor growth Growth	(Crutcher, 1989)
Savio	Neuroblastoma cells, neonatal rat DRG	White matter of adult rat brain, spinal cord and optic nerve	Poor Growth	(Savio and Schwab, 1989)

	neurons			
Carbenetto	Dissociated chick DRG neurons	Adult rat optic nerve and spinal cord	No growth	(Carbonetto et al., 1987)

Another interesting study of co-culture by (Savio and Schwab, 1989) demonstrated that grey matter, sciatic nerve and section of CNS white and grey matter were conducive substrate for the growth of the neuronal cells. The study was conducted to examine the substrate properties on neuronal growth. The experiments were conducted simply by using frozen CNS and PNS substrates for neuroblastoma cells and DRG. Overall, the results showed that adult rat CNS white matter; cerebellum, forebrain or optic nerve contained non-conducive properties and did not permit axonal regeneration and extension. This has been confirmed in other studies as presented in the table above where the growth of axons was relatively poor when grown on white CNS matter. However, our study illustrated that rat cerebellum and was quite permissive substrates for adhesion and neurite from adult zebrafish CNS neuronal cells.

Despite the presence of inhibitory molecules in rat CNS, the adult zebrafish neurons are still able to grow quite extensively on that environment. It has also been suggested (Becker et al., 1998a, Maier and Schwab, 2006) that developing appropriate experimental conditions for improving axonal regeneration is difficult to accomplish *in vivo* given that relatively few axons might actually be involved in the regenerative response and these are often difficult to discern amongst the mass of other axons present in CNS. In addition, the development of regenerating axons at

the site of lesion may possibly enhance their recovery and form appropriate synaptic connections (Aguayo et al., 1990). This study, therefore, combines two different species, adult zebrafish neurons and rat tissue substrate to examine neuronal regeneration.

The results from the present study did not support the hypothesis that adult zebrafish neurons would not be able to regrow their neurites on rat tissue substrate due to the non-conducive environment. The results suggest that adult zebrafish neurons are able to grow across the sections and form an extensive network of branches. The cryoculture technique is a convenient model that provides the examination of the growing of neurites on substrates. This model is very useful as it allows the observation of the complexities of events occurring *in vivo* to be circumvented while still providing the growing neurons with physiologically relevant substrata.

Importantly, neuronal growth occurred in a non-conducive environment, despite the presence of a number of factors that are known to inhibit such growth in mammalian neurons. Hence, neurite regeneration on rat tissue substrate might be possible due to its non-responsiveness of the neurons to the upregulation or release of inhibitory factors, which then down-regulates the growth of tropic factors and other physical barriers that impede the neurite regeneration in non-permissive environments. Adult zebrafish CNS neurons are known to be capable of regenerating their axons and forming synaptic connections with the right targets. The exact mechanism of why these lower vertebrates can regrow their axons after the injury is still unknown. However, it is possible that their ability to form stringent synaptic connections with other target molecules in the environment essentially promotes their growth (Tessier-Lavigne and Goodman, 1996, Scott and Luo, 2001).The presence of trophic factors

and other guidance molecules may be critical for the maturation of these neurons in the CNS in the presence of less or no inhibitory molecules.

Studies by (Bastmeyer et al., 1991, Bastmeyer et al., 1993) conducted similar experiments involving cross-species co-culture assay. The experiments were performed to investigate the interaction of growing gold fish retinal axons with rat oligodendrocytes co-cultures. Interestingly, it was found that the growth cones collapsed when gold fish axons encountered the rat oligodendrocytes and the axons failed to regenerate. However, the axons were able to grow around the surrounding area, but not across the rat oligodendrocytes. This experiment has illustrated that both mammalian neurons and the growing axons of fish retinal neurons are sensitive to myelin associated inhibitory proteins. However, in the presence of antibody IN-1 which was used to neutralise the inhibitory molecules, the axons responded to growth and were able to extend their branches across the mammalian oligodendrocytes (Chen et al., 2000, Bandtlow, 2003).

The findings from this study clearly suggested that the adult zebrafish neurons are able to grow on the rat tissue in small branches and become more visible when present on coated poly-lysine coverslips and non-tissue sections. This suggested the neurons did not mediate contact inhibition at the cell surface making them unresponsive to the receptors or ligands expression in the environment. Moreover, these adult zebrafish neurons might contain more growth factors, guidance molecules that block the release of inhibitory molecules, and other types of physical barriers. Lastly, the axons may not express receptor binding properties for any of the inhibitory molecules due to the evolutionary distance between them.

Reactive astrocytes in the CNS are thought to impede the axonal regeneration. The astrocytes might not act as a physical barrier to the newly growing neurons, but provide stop signals for growth which might possibly be mediated by anti-adhesive properties of molecules such as tenascin (Lotz et al., 1989). However, in some instances, astrocytes may support regeneration by releasing the diffusible factors such as fibroblast growth factor (Le and Esquenazi, 2002). A previous study by (Wehrle and Chiquet, 1990) indicated that tenascins exhibit both promoting and inhibitory roles on neurite regeneration. They may enhance the survival and growth of transplanted neurons (Apostolova et al., 2006). Furthermore, it has been revealed that astrocyte scars that formed in response to injury may lessen when exposed to x-irradiation. A study by (Pinjuh and Bedi, 2003) demonstrated that x-irradiation exposure of adult CNS tissue can modify the environment and increase the chance of neurite regeneration. This maybe one of the possible ways of making mammalian sections supportive of neurite regeneration.

One complication to this present study was the fact that there was a tendency for cryostat sections to become detached from the coverslips after about seven days in tissue culture. This was despite the presence of an adhesive coating such as poly-L-Lysine on the coverslips. In addition, the detachment of the sections may be due to the fact that the experiments were performed at incubation conditions that were suitable for fish culture which is at 28.5°C with 1.5% CO_2, as opposed to the normal mammal temperature at 37°C with 5% CO_2. Future investigations and more optimisations are required to prevent future detachment of the tissue sections from the adhesive substrates.

In conclusion, the present study has demonstrated that cryosections taken from adult rat cerebellum CNS tissues support the regeneration of neurites from adult zebrafish neurons *in vitro*. Successful regeneration was the main focus of this study. A clearer insight and deeper understanding of receptor-ligand interactions are required for future investigations in neuronal regeneration. Due to the time constraints, further experiments with different types of rat brain section were unable to be performed during the present project. However, these results indicate that further experiments are required to gain a full insight into adult CNS axonal regeneration. It would be useful to carry out further studies of co-culture on rat DRG and central neurons on cryostat sections taken from adult zebrafish brain in particular.

Moreover, experiments to determine whether adult zebrafish CNS neurons are capable of growing on specific regions (e.g. grey as opposed to white matter) of the mammalian CNS may also provide useful insights into the possible mechanisms of axonal regeneration. This would allow us to observe whether or not the neurons are repulsive to other areas of the rat brain substrate. In addition, rat DRG may be used to determine whether these cells are capable of growth on adult zebrafish brain tissue substrate and to verify whether the rat neurons will regenerate their neurite in the permissive environment of another species. It may also be necessary for future studies to examine whether combinations of neurons and other tissue substrates may improve regeneration. Examinations on whether blocking or removing inhibitory factors by using different blocking antibodies or specific agents such as enzymatic digestion (CSPGs) can promote the neuronal growth of zebrafish and rat neurons under different conditions are also recommended. This may be achieved by using antibodies such as Anti-Nogo or Anti-MAG. There is certainly a great potential for

important discoveries to be made in relation to removal of myelin inhibitory molecules in the non-permissive environment.

As shown in the results of this experiment, adult zebrafish can possibly extend their neurite over rat brain cryosection. Further studies may examine transplantation of adult zebrafish cultured neurons into the brain of a rat as this may encourage a further step into regenerating brain cells *in vivo* and differentiation. Zebrafish has an extensive ability to induce a regenerative response in all regions of the brain and contain many proliferation zones for neurogenesis throughout their lives. It may be possible to transplant adult zebrafish neurons into the proliferative zone of mammalian CNS to examine the reactivity of neurite growth (appendix 10). However, xenografting or transplanting of different species of animals would likely be fraught with difficulties including cross-species reactivity and tissue rejection (Palmer and Klein, 2003, Liu and Leach, 2011). The only possible approach to minimize these problems is to use immunosuppressive reagents to prevent the rejection of tissue and possibly the administration of growth factors to simulate the neurites proliferation and arborisation.

CHAPTER SEVEN

SUMMARY AND

GENERAL DISCUSSION

7.1 INTRODUCTION

It has long been known that many animal species are capable of repairing their bodies or major body systems, after injury (Sanchez Alvarado and Newmark, 1999, Brockes et al., 2001, Blanco et al., 2010). For example, certain species can regenerate new limbs or indeed considerable portions of the whole body after injury (Reyer et al., 1973, Bryant et al., 1981, D'Jamoos et al., 1998). When certain worms (e.g. platyhelminthes') are cut in half, the head end can regenerate a new tail end whilst the original tail can develop a new head (Sanchez Alvarado et al., 2002, Salo and Agata, 2012). This ability to repair the body after injury is present to some degree in all animals, including humans. However, the extent to which such repairs can be carried out appear to become more restricted in the higher species of animals compared to lower species (Stuermer et al., 1992, Sivron et al., 1994, Tsonis, 2000, Cameron et al., 2005, Tanaka and Reddien, 2011). Higher animals certainly do not retain the ability to grow a new head if the original is injured. Turning now to the central nervous system, it is known that many animals can also repair this crucial body system after injury, either by generating new neurons to replace ones that may have been lost, and/or by allowing injured neuronal axons to regenerate and establish new functional synaptic contacts with appropriate targets (Schwab and Bartholdi, 1996, Olson et al., 1998, Garcia and Koke, 2009). Thus, a severed goldfish or amphibian optic nerve can be spontaneously repaired so that vision is restored in the animal (Bastmeyer et al., 1991, Matsukawa et al., 2004). However, this remarkable ability to repair the adult central nervous system after injury appears to have been lost at the mammalian stage of evolutionary development. The question, therefore, arises regarding the exact changes which occurred at this stage

of evolution that caused this ability to be lost. A thorough understanding of the changes which occurred between these evolutionary stages may give an insight into the mechanisms of how we may repair the injured human brain or spinal cord (Herculano-Houzel, 2011). The main purpose of this research project was to investigate the cellular and molecular mechanisms during repair and re-growth of the axons after injury in lower vertebrate species. The adult zebrafish CNS is a model for lower vertebrates as it has the following properties (Barbazuk et al., 2000, Postlethwait et al., 2000, Lewis and Eisen, 2003, Guo, 2004):

- fast development, long lifespan and as well as easy to maintain
- similar CNS structure and cellular types as found in the mammalian brain

It was decided to examine the regenerative properties of adult zebrafish CNS neurons under various conditions. However, the major problem faced at the commencement of the project was that there were no previous published methods which could be used to isolate neurons from the adult zebrafish brain, therefore methods had to be developed to examine the conditions (chapter 3) under which it was best to grow and maintain such neurons in a tissue culture system.

Successful optimisation of culturing adult zebrafish CNS neurons was found to be dependent on important factors such as growth medium, incubation temperature, and the adhesive substrate used to coat the surface on which the neurons were grown (See chapters 3 and 5). Additionally, the presence or absence of neurotrophic factors on the growth of neurites within the tissue culture medium used was also studied (chapter 4). Finally, I investigated whether or not adult zebrafish CNS neurons were capable of regenerating axons when grown in a mammalian CNS

environment (chapter 6). I found that such neurons were indeed capable of regenerating their axons even in an environment which is known to be unsupportive of axonal regeneration from mammalian CNS neurons. The implication of this finding is that zebrafish CNS neurons may be unresponsive to the factors present in the CNS environment which inhibit axonal regeneration from mammalian neurons.

The contrasting failure of the lesion CNS neurons to regenerate their axons as opposed to the ability of injured lower vertebrate CNS neurons can be explained by the presence of potent growth inhibitory molecules present in the post lesion mammalian CNS environment (Bovolenta et al., 1993, Canning et al., 1996, Davies et al., 1999, Hermanns et al., 2001). As previously discussed (chapter 1), a lesion to the adult mammalian CNS causes a glial reaction which leads to the formation of glial scar at the site of the injury (Berry et al., 1983, Bahr et al., 1995). Such scar tissue characteristically recruits different cell types including microglia, astrocytes, oligodendrocytes and their precursors, and meningeal cells (Rasouli et al., 2009). These cell types subsequently divided, migrated and filled up the CNS space, creating a barrier which it is thought to inhibit axonal regeneration (Malhotra et al., 1995, Fawcett and Asher, 1999). In addition, other molecules are unregulated by many cell types in the environment which also cause the environment to be unfavourable for axonal regeneration. Several groups of such molecules have been identified and studied extensively including various components of myelin. These molecules include the neurite growth inhibitors associated with oligodendrocytes such as MAG, OMgp, Nogo, and proteoglycans including CSPG (Mckerracher et al., 1994, Ng et al., 1996, Busch and Silver, 2007, McCurley and Callard, 2010).

The capacity for CNS regeneration is fairly poor; therefore, the presence of only a few inhibitory molecules in the environment could easily cause an abortive regenerating response (Fawcett, 1992, Tetzlaff et al., 1994, Cai et al., 1999). Finding possible ways to overcome these inhibitory factors is a major goal of much worldwide research in this field. Recent studies have developed a few strategies that might be useful to counteract these inhibitory factors (Lu and Waite, 1999, Bunge, 2008). Common strategies that were thought to effectively minimize the damaged CNS were the application of blocking antibodies, removing receptors or neutralizing inhibitors (Keirstead et al., 1995, Fiedler et al., 2002, Fournier et al., 2002).

Reactive astrocytes within scar formation also have up-regulated CSPGs which potently restrain axonal regeneration (Brown et al., 2012). Various CSPGs such as neurocan, versican, and decorin are expressed after injury (McKeon et al., 1995). A study by (Stichel et al., 1999) showed that neutralising each component of the scar using Col III and IV and combining these with blocking antibodies such as IN-1 neutralizing antibody could significantly increase axonal outgrowth (Ferraro et al., 2004, Klapka et al., 2005). In some instances, hyaluronidase treatment could also be applied to cleave CSPGs rendering them less inhibitory (Moon et al., 2003, Del Rio and Soriano, 2007). In addition, it was recently suggested that chABC treatment might improve the capacity for axonal outgrowth as the enzyme chondroitinase digested CSPGs in the glia scar, rendering the environment to be more permissive and supportive of the regeneration (Rasouli et al., 2009). In other experiments, it has been shown that x-irradiation might be a strategy that can suppress the lesion scar formation as well as decrease the expression of CSPGs (Zhang et al., 2005b). Various blocking antibodies have also been used to neutralise myelin associated

inhibitors (Schwab, 2004). It was shown that the inhibitory activities that triggered Nogo-A could be effectively removed by IN-1 monoclonal antibody (Caroni and Schwab, 1988, Fournier et al., 2002). Treatment with such antibodies improved axonal sprouting and functional recovery (Bregman et al., 1995, Thallmair et al., 1998). It may be possible to use one or more of the above strategies to further improve the extent of axonal regeneration from adult zebrafish CNS neurons on various substrates that would normally be unsupportive of mammalian regeneration.

One of the aims in this present study was to examine the effects of altering the CNS environment to promote favourable growth. It was believed that the permissive condition within the CNS is achievable when the function between promoting molecules and those that block the axonal regeneration are balanced. It was surprising to see that lower vertebrate species such as adult zebrafish neurons could regenerate their axons on the surface of highly inhibitory rat cerebellum tissue sections. This result poses some important questions regarding the presence or absence of inhibitory factors or their receptors on the surface of adult zebrafish CNS neurons. It is suggested that further research in this area may yield some important findings regarding the nature of substrate neuronal cell interactions. Axonal regeneration can also be enhanced by introducing growth promoting molecules as well as removing or blocking inhibitory factors. Neurotrophic factors are able to improve the survival of neurons after injury and promoting their axonal regeneration. Many neurotrophic factors also can act as neurotrophic agents and guide regenerating axons to their correct target sites (Xu et al., 1995, Stichel and Muller, 1998). In this study, the effect of various neurotrophic factors on the extent of neurite regeneration from adult zebrafish CNS neurons was examined. It was clearly shown

that by inducing the neuronal cells with certain concentrations of neurotrophic factors, the growth could be significantly enhanced. It was also noted that neurotrophic factors may depend on mitogenic factors to encourage differentiation during development of neurons (Renaud et al., 1996). Critical examinations in terms of cell survival, differentiation and their activity in neuronal development are required in order to achieve the best growth culture and possibly provide clinical applications in promoting growth within mammalian CNS (Davies, 1995). This section of this study is only a small part of the global effort in determining how to repair the CNS after trauma injury.

7.2 FUTURE DIRECTIONS

In regards to advancing the findings of these present studies, additional clinical investigations involving the following would indeed open up a new way to promote axonal regeneration (Cotman et al., 1984, Bregman et al., 1997, Horner and Gage, 2000, Reier, 2004, Bunge, 2008);

- neurotrophic factors delivery
- pathway that regulate both promoting and inhibitory molecules
- gene modification therapy
- cell grafting
- application of neural stem cell to transplant and replace the injured cell
- remyelination process in transgenic zebrafish using *in vivo* imaging

To date, the application of neural stem cells has turned out to be one of the most promising strategies to repair the injured mammalian CNS (Murphy et al., 1997, Reier, 2004, Vogel, 2005). The unique characteristic of these cells is the ability to differentiate and self-renew into a restricted subset of other cell types (Flax et al., 1998, Gage, 2000). Recent studies are focusing on the isolation and proliferation of stem cells that can be transplanted directly to the injured region without any rejections (Bartlett, 1982, Jiang et al., 2008).

In parallel to these neuroregenerative treatment studies, it would be important to examine the neuroprotection strategies which are the potential factors that avoid excessive neuronal cell death in the CNS after trauma. This might be one of the possible approaches to enhance the recovery of neuronal function after CNS damage (Gurgo et al., 2002, Hall and Springer, 2004).

7.3 OUTCOME AND SIGNIFICANCE

In conclusion, the overall outcome of this current study suggested that the adult zebrafish was an excellent model to examine neural regeneration and CNS development. Furthermore, these results provided evidence that neurotrophic factors and cell adhesion substrates facilitated the regeneration of neuronal axons in the tissue culture system used. The fact that adult zebrafish neurons could grow and extend their neurites on substrates that are non-conducive, to such growth for mammalian neurons, could be of great significance. The exact reasons for these differences between mammalian and zebrafish CNS neurons need to be examined further and may give insights into how we may repair the injured mammalian CNS.

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APPENDICES

APPENDIX 1

Animal weights and the usage in three years

Animals	Number of Animals used
Adult zebrafish	428
Rats	22

AEC Reference Number: CA 2008/07/278

APPENDIX 2

Components of modified Bottenstein and Sato are medium with 2% foetal bovine serum

Components	Sources	Concentration	Volume
Transferrin	Sigma	10 mg/ml in Ham F -12	1 ml
Bovine Serum Albumin	Sigma	300 mg/ml essentially fatty acid free	1 ml
Foetal Calf Serum	Sigma	N/A	2 ml
Hams F-12 medium	Sigma	1X	94 ml
Penicillin/Streptomycin	Gibco BRL	10 000 ug/ml	1 ml
Progesterone	Sigma	60 ug/ml in 95% ethanol	100 ul
Putrescine	Sigma	1.6 mg/ml in Hams F12	1 ml
Sodium Selenite	Sigma	1.6 mg/ml in Hams F12	10 ul
Insulin	Sigma	10 mg/ml in 1% glacial acetic acid	100 ul

Preparation of Modified Bottenstein and Sato's Medium with 2% Foetal Calf Serum (BSF2)

1) The constituents of BSF2 tabulated above were carefully mixed under sterile conditions in a 200 ml conical flask, making sure the insulin was added last.

2) The medium was adjusted to a final pH of 7.1 using ~ 100µl of 1M HCl.

3) Media was carefully drawn into a 60cc syringe (Terumo Tokyo, JPN) and filter sterilised through a 0.22µm filter unit (Millipore Bedford, MA) into an autoclaved bottle.

4) BSF2 was stored at 4°C and discarded if not used within ~ 3 weeks.

APPENDIX 3

Preparation of paraformaldehyde fixative (4% PFA in 0.1 M phosphate buffer

In order to make this solution, ½ the required final volume of 8% PFA was added to ½ the required volume of 0.2 M phosphate buffer.

1) Weigh out the required amount of paraformaldehyde to make an 8% solution.

2) Add this paraformaldehyde to distilled water, so that the volume comprises ½ the final required volume of fixative.

3) Add to this beaker the same volume of 0.2M phosphate buffer.

4) Cover the beaker with parafilm, stir and heat under a fume hood until the solution reaches a temperature of approximately 65°C (Do not allow the temperature to exceed 70°C)

5) Use a solution of 1M NaOH to adjust the pH of the fixative. The required volume of NaOH is approximately the same in µl as the final volume of fixative in ml (ie. If the volume of fixative is 100 ml, add approximately 100µl of 1M NaOH.

6) Allow the paraformaldehyde to dissolve. The solution will be entirely translucent.

7) Check the final pH of the fixative and adjust if necessary

8) Cool fixative on ice before use.

APPENDIX 4

0.2M phosphate buffer saline (PBS)

1) To make 2 litres, add 42.6g of disodium hydride orthophosphate to 1500mL of deionised water.

2) Add 12.5g of sodium dihydride orthophosphate to 400ml of deionised water in another beaker.

3) Stir both separately on hot plates until all the solute is dissolved.

4) Slowly add the dihydride to the disodium until a pH of ~7.5 is attained. Stored at a room temperature.

APPENDIX 5

The protocol for immunohistochemical staining of adult zebrafish neurons

1) Fix in 2% paraformaldehyde for few minutes.
2) Wash 3 X in PBS
3) Fix in cold methanol (-20ºC)
4) Wash 3 X in PBS
5) Incubate in rabbit polyclonal anti GAP-43 (Zebrafish specific) [1:200, 1:500]
6) Wash 3 X in PBS
7) Incubate in Goad anti Rabbit IgG conjugated with cy [1:500]
8) Wash 3 X in PBS
9) Fix in cold methanol (-20ºC)
10) Wash 3 X in PBS
11) Wash 3 X in distilled water
12) Mount in 90% glycerol in 10% PBS containing a few granules of Diazabicyclo (DABCO)

APPENDIX 6

The protocols for immunohistochemical double staining (adult zebrafish neurons on CNS cryosection

1) Fix in 2% paraformaldehyde
2) Wash 3 X in PBS
3) Fix in cold methanol (-20ºC)
4) Wash 3 X in PBS
5) Incubate in rabbit polyclonal anti GAP-43 for 3 hours [1:500]
6) Wash 3 X in PBS
7) Incubate in monoclonal mouse anti GFAP for 3 hours [1:500]
8) Wash 3 X in PBS
9) Incubate in Goat anti Rabbit IgG conjugated with cy2 [1:500] for 1 hour
10) Wash 3 X in PBS
11) Incubate in Goat anti IgG conjugated with cy3 or FTTC
12) Wash 3 X in PBS
13) Fix in cold methanol (-20ºC)
14) Wash 3 X in PBS
15) Wash 3 X in distilled water
16) Mount in 90% glycerol in 10% PBS containing a few granules of Diazabicyclo (DABCO)

APPENDIX 7

a) Antibody Diluent

1) To make 100 ml, measure out 0.5g of Bovine serum albumin (Sigma), 0.5g of Saponin (Sigma) and 0.5g of sodium azide.
2) Add this to 100ml of PBS and stir until dissolved.
3) Store the solution at 4°C.

b) Anti-Fade Mountant

1) Heat 90ml of glycerol (Sigma) in a water bath of 37°C.
2) Add 2.5 of (1,4)-diazbicyclo-(2,2,2)-octane (DABCO;BDH), stirring slowly until completely dissolved.
3) Add 10ml of PBS and stir until combined.
4) Store at 4°C and heat in a 37°C water bath before use.

APPENDIX 8

P-values of coated coverslips (chapter 5)

Substrate	*P-Values*
Fibronectin+gelatin	0.027 -> significant
Poly-L-lysine	0.081
Poly-D-lysine	0.293
Laminin	0.140
collagen	0.442
Poly L-lysine + Laminin	0.003

P-values of glass and plastic coverslip

=0.035 (significant)

ANOVA result is reported as an F statistic and its associated degrees of freedom and p value.

P-value: a Test of Significance <0.05

F-value: Measurement of distance between individual distributions

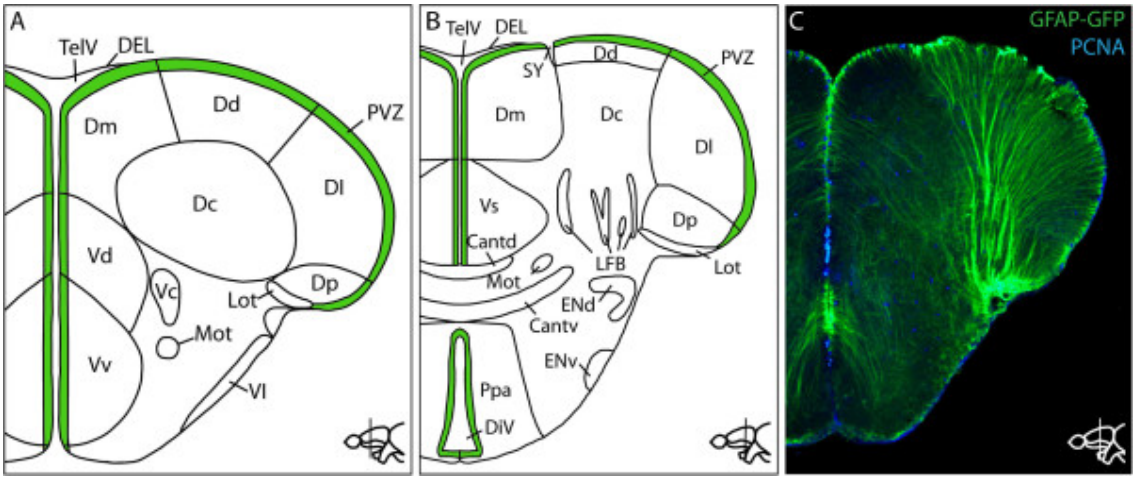
(Mean Square of X / Mean Square of Error)

APPENDIX 9

Role of Various Neurotrophic Factors in Neuronal Development Maturation and Differentiation

Stage of development	Neurotrophic factors	Actions
Proliferation of precursors	BDNF, NT-3, bFGF	Differentiation
Target contact by immature neurons	BDNF, NT-3, NT-4/5	Survival maturation
Synapse elimination	BDNF, NT-4/5, CNTF, FGFs, IGFs	Sprouting synapse stabilization
Functional maturation	BDNF, NT-3, NT-4/5, CNTF	Synaptic efficacy

APPENDIX 10



A number of new neurons produced in many areas unlike mammalian brain, this figure illustrated the anatomy of the telencephalon region of adult zebrafish (one of the proliferative and neurogenesis zone) Micrograph a) demonstrated a transverse section through the anterior part and b) posterior section. The proliferative zones are clearly specified in green. C) Showed the immune-staining of proliferative zone.